2. MATERIALS, EXPERIMENTAL, RESULTS & DISCUSSION

2.1 EXPERIMENTAL DESIGN

The objective of this investigation was to develop and evaluate an improved ophthalmic dosage form of ciprofloxacin HCl, for the treatment of bacterial keratitis and conjunctivitis. Therefore, the following experimental plan was adopted. The first phase of the experimental work involved preformulation studies as well as development and evaluation of conventional eye-drops and eye ointment containing ciprofloxacin HCl. These formulations are available commercially but the technology for their manufacture is not revealed.

An extensive literature search revealed that the development of a long acting aqueous gel or a formulation additionally containing an anti-inflammatory drug, such as a steroid, could significantly improve the therapeutic efficacy of ciprofloxacin HCl applied topically to the eye. Hence, in the second phase of the study, experiments were designed to develop a long acting gel formulation. In the third phase of the study, investigations were directed to develop eye drops containing a combination of ciprofloxacin HCl and dexamethasone, a potent steroidal anti-inflammatory drug.

Experiments were initiated with preformulation studies. The first step in the preformulation studies was to develop and/or standardize analytical methods for assay of the drugs. The official analytical methods and those published in the literature, for the drugs under investigation, were standardized to our laboratory conditions. Analytical methods were

standardized for assay of ciprofloxacin from bulk drug and also in the presence of proposed formulation additives. The next step was to standardize the bulk drug samples and the additives. Hence ciprofloxacin HCl, dexamethasone and a few of the additives were standardized as per their respective official monographs, so as to ensure their compliance to official standards.

Further experiments were designed, with an aim to: (i) determine drug-additive compatibility, (ii) select suitable containers for dispensing the formulations, (iii) establish methods of sterilization of ciprofloxacin HCl and dexamethasone powders as well as their aqueous solutions.

With inputs from the preformulation studies, additives were short-listed, suitable containers were selected and the following formulations containing ciprofloxacin HCl alone were prepared:

i) 0.3% w/v ciprofloxacin ophthalmic solution (**Cip. solution.**)

ii) 0.3% w/w ciprofloxacin ophthalmic ointment (**Cip. ointment**)

iii) 0.3% w/w ciprofloxacin ophthalmic gel (long-acting) (**Cip. gel**)

While preparing the formulations, the emphasis was on development of a process which could be adapted to large-scale manufacture. The simplest formula, with only the essential additives was used so as to make the formulations cost effective.

In order to prepare the long-acting gel formulation, Poloxamer-407, a unique polymer that confers thermoreversible properties on the gel was chosen. This gel liquefies on cooling and gels at body temperature, hence it offers the advantage of being filled

and dispensed as a solution, on cooling and gelling on instillation into the eye, from where the drug would be released in a controlled manner. The polymer concentration to be used in the formulation was also optimized.

Dexamethasone was chosen because of its high anti-inflammatory potency and lack of mineralocorticoid activity. Dexamethasone sodium phosphate was initially chosen to be incorporated in the formulation as it is highly water soluble, however, it being an alkaline salt was found to be incompatible with ciprofloxacin HCl, hence dexamethasone as such was used. However, since dexamethasone is insoluble in water, an aqueous suspension dosage form was designed. Recently, there have been reports on the utilization of hydroxypropyl-β-cyclodextrin to solubilize dexamethasone for use in parenteral as well as ophthalmic solutions. Therefore, an additional formulation was prepared in which dexamethasone was solubilized with hydroxypropyl-β-cyclodextrin. Thus, the following two formulations containing ciprofloxacin HCl and dexamethasone were developed and evaluated:

i) 0.3% w/v ciprofloxacin and 0.1% w/v dexamethasone ophthalmic suspension (**Cip + Dexa suspension**)

ii) 0.3% w/v ciprofloxacin and 0.1% w/v dexamethasone ophthalmic solution (**Cip + Dexa solution**)

Suitable *in-vitro* and *in-vivo* methods were designed and selected to evaluate the developed formulations, which included physicochemical, microbiological and biological tests.

In order to determine the quality of the products, the prepared formulations were subjected to some typical tests such as assessment of clarity/resuspendability, pH of solution, determination of particle size, determination of viscosity as well as *in-vitro* drug release profile. Accelerated stability studies were conducted for a period of 6 months, at 5°C, 25°C, 37°C, 45°C, 37°C/75%RH and also under accelerated conditions of light. The drug-s were assayed by the stability-indicating HPLC method at intervals of 1,2,3,5 and 6 months. The preservative was also assayed at the beginning as well as at the end of stability studies. Microbiological tests applicable to sterile products, such as, test for sterility and test for preservative efficacy were also conducted.

The products were also evaluated ***in-vivo*** in rabbit eyes. Topical ophthalmic products have to be evaluated for their potential to cause eye-irritation upon instillation/application. The standard test recognized by the USFDA for this purpose is the Draize test. Thus, the eye-irritation potential of the prepared formulations was studied in healthy New Zealand rabbits, following the guidelines of the Draize test.

The efficacy of ciprofloxacin containing formulations could be directly related to its concentrations in the ocular tissues. The aqueous humour is in dynamic equilibrium with the corneal tissue and hence can be sampled to assay drug which would reflect the concentration in the cornea, the target tissue. Hence, a reported HPLC method was standardized to assay ciprofloxacin from the aqueous humour in nanogram quantities. The study involved

instillation of a single dose or multiple doses, withdrawing the aqueous humour over a period of time and assaying it for ciprofloxacin. Thus, the aqueous humour drug concentration - time profile was determined after: (i) instillation of single dose of the prepared conventional solution and compared with that of its marketed counterpart, (ii) instillation of single dose of the gel and compared with multiple doses of the conventional solution and (iii) application of a single dose of the ointment and comparing it with multiple doses of the solution.

In order to demonstrate the efficacy of the ciprofloxacin + dexamethasone combination, an animal model of severe bacterial keratitis induced corneal neovascularization was developed. The ability of the combination to prevent neovascularization vis-a-vis ciprofloxacin conventional solution as well as saline treated controls was demonstrated.

2.2 MATERIALS:

Drugs and additives:

Benzalkonium chloride 50% solution: Spectrochem, India

Boric acid AR: Qualigens

Carbopol 940 and 971P: B.F. Goodrich Co, USA

Cholesterol GR: Loba Chemie, India

Ciprofloxacin HCl USP: Courtesy Ranbaxy Laboratories Ltd., India

Ciprofloxacin HCl USP (micronized): Courtesy Cipla Ltd., India

Ciprofloxacin ethylenediamine analog A USPRS: Courtesy Cadila Pharma, India

Dexamethasone IP (micronized): Avik Laboratories Pvt. Ltd., India

Dextrose AR: Qualigens, India

Disodium EDTA AR: Qualigens, India

Fluoroquinolonic acid USPRS: Courtesy, Dr. Reddy's Laboratories, India

Glacial acetic acid AR: Qualigens, India

Glycerol IP: United Chemicals, India

HPMC E4M: Colorcon, UK

Hydroxypropyl-β-cyclodextrin: Wacker Chemie, Germany

Mannitol, puriss: Spectrochem, India

Poloxamer-407 NF: BASF, Germany

Propylene glycol AR: S.D. Fine Chemicals Ltd. India,

Sodium acetate trihydrate AR: Qualigens, India

Sodium chloride AR: Qualigens, India

Sodium metabisulfite LR: S.D. Fine Chemicals Ltd., India

Thiomersal: National Chemicals, India

α-tocopherol: BASF, Germany

Tween 80: Prolabo, France

Water: Freshly double distilled water from an all-glass distillation still

White soft paraffin IP: Omega Labs., India

Apparatus, equipment etc.

Aluminium crimps: M/s Jayantilal Mehta & Sons., India

G-4 filter: Borosil, India

Grey butyl rubber stoppers 20mm: Shree products, India

Membrane filters 0.22 µm (GV): Millipore

Presterilized LDPE stoppers: Vijay Bakelite, India

pH meter: Elico, model: LI-120, India

pH meter electrode: Elico, model CL-51, India

Type-I glass vials: Gujarat glass, India

Solvents and chemicals used for analysis:

Absolute alcohol (Aldehyde free alcohol, AFA): Alembic Chemical Works Co. Ltd., India

Acetonitrile AR: S.D. Fine Chemicals, India

Ammonia solution sp. gr. 0.91, AR: Qualigens, India

Chloroform AR: Qualigens, India

Dioxan, spectroscopy grade: Spectrochem, India

Heptanesulphonic acid sodium salt, chromatography grade: Spectrochem, India

Hydrochloric acid AR: Qualigens, India

Hydrogen gas, IOLAR-1: Indian Oxygen Ltd., India

Karl Fischer reagent: Qualigens, India

Kieselguhr G, for TLC: S.D. Fine Chemicals Ltd., India

Litmus indicator powder: Qualigens, India

Methanol AR: Qualigens, India

Methylene chloride AR: S.D. Fine Chemicals Ltd., India

Mercuric chloride AR: Qualigens, India

Nitric acid AR: Qualigens, India

β-naphthol, pro analysi: Prolabo, France

Nitrogen, zero air: Poonam Oxygen Ltd., Ahmedabad, India

Orthophosphoric acid AR: S.D. Fine Chemicals, India

Phenylhydrazine: Spectrochem, India

Potassium bromide, *zur Analyse*: Merck AG, Darmstadt, Germany

Potassium dihydrogen orthophosphate AR: Qualigens, India

Potassium iodide AR: S.D. Fine Chemicals Ltd., India

Potassium iodate AR: S.D. Fine Chemicals Ltd., India

Potassium nitrate AR: Qualigens, India

Potassium permanganate LR: S.D. Fine Chemicals, India

n-propyl alcohol AR: S.D. Fine chemicals Ltd., India

Silica gel GF254: Merck, India

Silver nitrate AR: BDH Chemicals, India

Sodium dihydrogen orthophosphate AR: Qualigens, India

Sodium hydroxide AR: Qualigens, India

Sodium nitrite AR: Qualigens, India

Solvent ether LR: Qualigens, India

Sulphuric acid AR: S.D. Fine Chemicals, India

Tetrabutylammonium hydroxide AR: Sisco Research Laboratories, India

Tetrahydrofuran, HPLC grade, stabilized: Spectrochem, India

10% Tetramethyl ammonium hydroxide solution: Merck, India

Tetrazolium blue: Loba Chemie, India

Water: Freshly double distilled water from an all-glass distillation still

Zinc powder: S.D. Fine Chemicals Ltd., India

Instruments used for analysis:

* Analytical balance: Mettler AE 240, Switzerland

* Differential Scanning Calorimeter: Perkin Elmer, model DSC7, USA

* Gas Chromatograph: Chemito, model 8570, India

  - Column: Porapak Q, 1.5m X 4mm i.d.

  - Data processor: Chemito, model 5000, India

  - Detector: Flame ionization type

* HPLC:

  - Column: Phenomenex-Bondclone, C_{18}, 10µm, 15cm X 3.9mm i.d.

  - Guard column: Waters, C_{18}, 35-45µm Corasil^{R}, 3cm X 4 mm i.d.

  - Fluorescence detector: Jasco, model FP-920, Japan

  - Injector: Rheodyne 7125, with 20µL loop

  - Integrator: Chromatocorder 21, System Instruments Co. Ltd. Japan

  - Solvent delivery pump: Jasco, model 880-PU, Japan

  - UV-Vis detector: Jasco, model 875-UV, Japan

* Infrared spectrophotometer: Buck Scientific, model 500, USA

* Light microscope: Leitz, Laborlux 12, Germany

* pH meter: Elico, model LI-120, India

* pH meter electrode: Elico, model CL-51, India

* Polarimeter: Jasco, model DIP-360, Japan

* UV light cabinet: Camag, UV cabinet II, Switzerland

* UV-Visible spectrophotometer (double beam): Jasco, model 7850, Japan

* Viscometer: Brookefield, type LVDV-II+, Brookefield Instruments Inc., USA

Materials used in microbiological studies:

Bacteriological agar: Qualigens, India

Beef extract: Qualigens, India

Fluid thioglycollate medium: Himedia, India

Membrane filters (0.22µm, GV): Millipore,

Microbial cultures: Courtesy, Cadila Pharma

Peptone, bacteriological: S.D. Fine Chemicals Ltd., India

Polysorbate 80, for bacteriology: Himedia, India

Soyabean casein digest medium: Himedia, India

Drugs used in animal studies:

Ketamine hydrochloride: Ketmin^{R} injection, 50 mg/mL, Themis Pharmaceuticals Ltd., India

Xylazine hydrochloride: Courtesy, Cadila Pharma, India

Xylocaine: Lidocaine^{R} topical, 4% w/v solution, Astra-IDL, India

2.3 PREFORMULATION STUDIES:

The preformulation studies were broadly divided into the following groups:

2.3.1 STANDARDIZATION OF ANALYTICAL METHODS:

The first part of the preformulation work was to develop or standardize suitable analytical methods for assay of the drug-s. Analytical method development and its validation are an integral part of the product development programme. The type of analytical method selected depends largely upon, the purpose of assay eg. drug content, stability studies, dissolution studies; as well as other factors such as concentration of the drug to be assayed, the sensitivity required and the type of matrix from which the drug is to be assayed. Besides assaying the drug from ophthalmic dosage forms, the USP also recommends assay of the preservative, which is vital to the product stability. The following analytical methods were standardized in our laboratory:

2.3.1.1 METHODS FOR THE ASSAY OF CIPROFLOXACIN HCl:

(A) Standard plot for assay of ciprofloxacin from formulations:

The methods for analysis of drugs from formulations were standardized by studying their recoveries from solutions containing mixtures of proposed additives and also confirming their lack of interference. A part of this work is dealt with in Section-2.2.1.2, where drug-additive compatibility studies are reported. These methods were later adapted for analyzing drug from the final formulations. Hence, the results of the method

standardized for the final formulations are reported herein.

EXPERIMENTAL:

The official HPLC method for the assay of ciprofloxacin from ophthalmic solution is itself a stability-indicating method and hence this method was standardized to our working conditions. The column used was C_{18} (details in 'Materials'). The mobile phase consisted of 0.005M tetrabutylammonium hydroxide, adjusted to pH 2 with orthophosphoric acid : methanol (75:25 %v/v). The mobile phase was pumped at a flow rate of 1mL/min and the eluent was detected by means of an ultraviolet detector at 280nm. The injection volume was 20µL. The number of theoretical plates on the column was not less than 1500. Standards corresponding to 50, 60 and 75 µg/mL ciprofloxacin were injected along with the authentic degradation product, ciprofloxacin ethylenediamine-analog (Analog-A). Thus, a 3-point standard curve was constructed for assay of ciprofloxacin. Recoveries of the drug were calculated at three different concentrations, namely 110%, 100% and 90% of the drug concentration in the formulation.

RESULTS & DISCUSSION:

The chromatographic separation of ciprofloxacin and its principal degradation product Analog-A is shown in Fig. 3.

(A) Standard plot for assay of ciprofloxacin from formulations:

The equation of the regressed line was :

$Y = 173252.771\ X - 49253.38 \quad n = 6, \quad r = 0.9981$

The coefficient of correlation, r, shows excellent correlation between concentration and the peak areas. The percentage accuracy

was within 1.5%, which shows that the standard plot can be used to back calculate the concentrations accurately. The %CV (percentage coefficient of variation) between the areas was found to be not more than 0.75% which is quite low suggesting good precision of measurement.

The results of the recovery studies carried out at different concentrations from the formulations were as shown in Tables-1-3:

TABLE-1: RECOVERIES OF CIPROFLOXACIN HCl FROM CIPROFLOXACIN OPHTHALMIC SOLUTION:

% of drug present	Mean % recovered (n=6) (Intra-day)	Intra-day %CV (n=6)	Mean % recovered (n=12) (Inter-day)	Inter-day %CV (n=12)
90	98.96	0.85	98.05	0.92
100	98.98	0.98	98.78	0.97
110	98.07	1.08	99.16	0.92

TABLE-2: RECOVERIES OF CIPROFLOXACIN HCl FROM CIPROFLOXACIN OPHTHALMIC GEL:

% of drug present	Mean % recovered (n=6) (Intra-day)	Intra-day %CV (n=6)	Mean % recovered (n=12) (Inter-day)	Inter-day %CV (n=12)
90	99.51	0.82	99.67	0.90
100	99.15	1.00	99.37	1.06
110	99.52	0.79	99.54	0.95

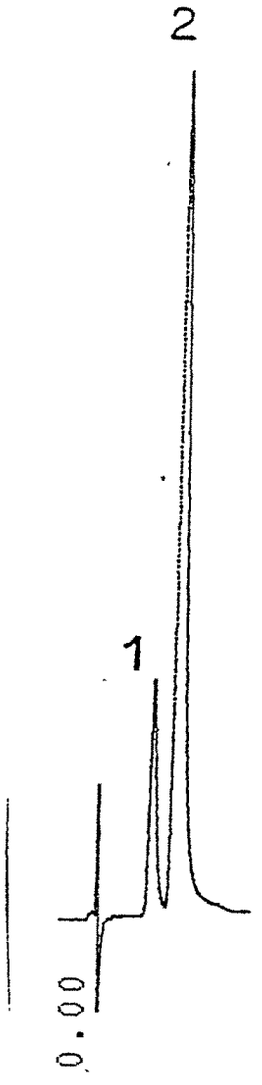

Fig. 3: Chromatogram showing separation of
(1) Ciprofloxacin ethylenediamine analog (Analog-A)
and (2) Ciprofloxacin

TABLE-3: RECOVERIES OF CIPROFLOXACIN HCl FROM CIPROFLOXACIN OPHTHALMIC OINTMENT:

% of drug present	Mean % recovered (n=6) (Intra-day)	Intra-day %CV (n=6)	Mean % recovered (n=12) (Inter-day)	Inter-day %CV (n=12)
90	99.67	1.42	100.30	1.67
100	98.66	2.21	98.85	1.94
110	98.56	2.06	99.02	1.97

The results indicated that the recoveries were close to 100% and were consistent, within day as well as between days.

2.3.1.1 (B) Standard plot of ciprofloxacin from (1) dissolution medium, phosphate buffered saline (PBS), pH 7.4 and (2) in 0.1N HCl for assay of ciprofloxacin from ointment for, content uniformity:

EXPERIMENTAL:

11.65mg ciprofloxacin HCl USP, (corresponding to 10mg ciprofloxacin base) was accurately weighed and was transferred to a 100mL volumetric flask. This was dissolved in about 5mL of distilled water and rest of the volume was made up with PBS. Similarly, 11.65mg of the drug was weighed and transferred to another 100mL volumetric flask, the powder was dissolved in about 10mL of 0.1N HCl and volume was made upto the mark with more 0.1N HCl. A series of dilutions were made so as to obtain solutions of the following concentrations: 1, 2, 4, 6 and 8µg/mL. The absorbances of the standard solutions were read on a double beam spectrophotometer, at 274nm for PBS and 277nm for 0.1N HCl.

RESULTS & DISCUSSION:

(B) Standard plots of ciprofloxacin in 0.1N HCl and PBS:

(1) Standard plot of ciprofloxacin HCl in 0.1N HCl:

The equation of the regressed line was:

Y = 0.1225 X - 0.0016, n=16, including (0,0), r = 0.999977

The %CV between absorbances was found to be less than 1%, suggesting good precision of the method. Each concentration could be back calculated to within 1.5% of its actual value, which suggests accuracy of the method.

(2) Standard plot of ciprofloxacin in dissolution medium (Phosphate Buffered Saline IP, pH 7.4), at 274 nm:

The equation of the regressed line was:

Y = 0.1189 X - 0.0020, n=16, including (0,0), r = 0.999974

The %CVs between areas were less than 0.5%, at all concentrations. Each concentration could be calculated back with an accuracy of ±0.5% of its actual value, which suggests that the method was accurate.

2.3.1.1 (C) Standard plot for estimation of ciprofloxacin from aqueous humour:

EXPERIMENTAL:

The HPLC method reported by Basci *et al*[231] was standardized to our working conditions. 11.65mg of the drug was weighed and transferred to a 100mL volumetric flask. The drug was dissolved in sufficient distilled water to make 100mL. From this solution, 1mL was further diluted to 100mL with distilled water. This gave

a working stock solution of 1µg/mL. The working stock solution was used to make standard solutions in distilled water in a concentration range of 10ng/mL to 500ng/mL.

(C) RESULTS & DISCUSSION:

The equation of the regressed line was :

$Y = 3111.73 X + 5601 \quad n = 12 \quad r = 0.99988$

The %CVs between areas were less than 5%, and the accuracies at different concentrations were better than 5%. These results suggest good accuracy and precision of the method since the acceptable limits of accuracy and precision in case of biological samples are ±10%. Since the method reports almost 100% recovery of ciprofloxacin from the aqueous humour, the authors suggest that simple aqueous solutions can be used to prepare standard curves rather than using aqueous humour spiked with ciprofloxacin.

2.3.1.2 METHODS FOR THE SIMULTANEOUS ASSAY OF CIPROFLOXACIN AND DEXAMETHASONE:

Standard plot for assay of ciprofloxacin and dexamethasone from formulations:

EXPERIMENTAL:

The official (USP XXIII) stability-indicating HPLC method for assay for dexamethasone from its ophthalmic suspension was slightly modified. The column used was C_{18}. The mobile phase consisted of 10mM heptanesulphonic acid adjusted to pH 3 with orthophosphoric acid : methanol (50:50 v/v) which was pumped at a flow rate of 1mL/min. The standards were made in a 1:1 mixture

of methanol:double distilled water. The eluents were detected by means of an ultraviolet detector at 254nm. The injection volume was 20µL. A 3-point standard curve was developed which corresponded to 125, 150 and 187.5µg/mL of ciprofloxacin and 41.67, 50 and 62.5µg/mL of dexamethasone. Recoveries of both the drugs were calculated at three different concentrations, namely, 110%, 100% and 90% of the drug concentrations.

RESULTS & DISCUSSION:

Standard plot for simultaneous estimation of ciprofloxacin and dexamethasone from formulations:

The chromatographic separation of ciprofloxacin, dexamethasone and Analog-A are shown in Fig. 4.

(A) CIPROFLOXACIN:

The equation of the regressed line was :

$Y = 593967.16 X - 2473336.83$, $n = 6$, $r = 0.999$

The %CVs at each concentration were less than 1.5% and the accuracies were within a limit of ±0.5%.

(B) DEXAMETHASONE:

The equation of the regressed line was :

$Y = 11405.20 X - 21620.38$, $n = 6$, $r = 0.9991$

The %CVs at each concentration were less than 1.5% and the accuracies were within a limit of ±1.0%.

The recoveries of ciprofloxacin and dexamethasone from the two preparations were as presented in Tables 4-7:

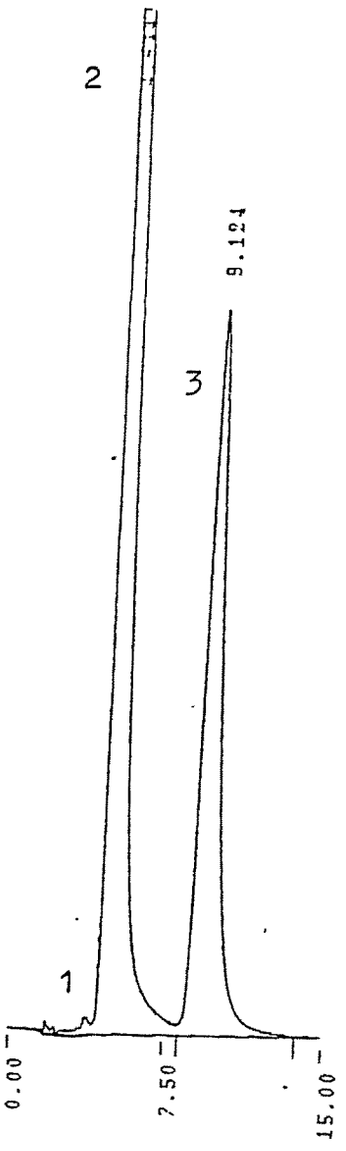

Fig. 4: Chromatogram showing separation of (1) Analog-A, (2) Ciprofloxacin and (3) Dexamethasone

TABLE-4: RECOVERIES OF CIPROFLOXACIN FORM CIP + DEXA SUSPENSION:

% of drug present	Mean % recovered (n=6) (Intra-day)	Intra-day %CV (n=6)	Mean % recovered (n=12) (Inter-day)	Inter-day %CV (n=12)
90	99.26	1.43	99.55	1.48
100	98.90	1.20	99.25	1.12
110	100.17	1.72	99.59	1.58

TABLE-5: RECOVERIES OF CIPROFLOXACIN FORM CIP + DEXA SOLUTION:

% of drug present	Mean % recovered (n=6) (Intra-day)	Intra-day %CV (n=6)	Mean % recovered (n=12) (Inter-day)	Inter-day %CV (n=12)
90	98.63	1.79	98.74	1.12
100	98.22	1.33	99.44	1.94
110	98.51	1.73	98.78	1.79

TABLE-6: RECOVERIES OF DEXAMETHASONE FORM CIP + DEXA SUSPENSION:

% of drug present	Mean % recovered (n=6) (Intra-day)	Intra-day %CV (n=6)	Mean % recovered (n=12) (Inter-day)	Inter-day %CV (n=12)
90	94.77	1.07	94.51	1.26
100	94.40	0.56	94.09	0.84
110	93.84	1.25	93.99	1.27

TABLE-7: RECOVERIES OF DEXAMETHASONE FROM CIP + DEXA SOLUTION:

% of drug present	Mean % recovered (n=6) (Intra-day)	Intra-day %CV (n=6)	Mean % recovered (n=12) (Inter-day)	Inter-day %CV (n=12)
90	94.51	1.71	94.46	1.35
100	94.03	0.89	94.14	1.05
110	93.70	0.87	93.55	1.00

The recoveries of ciprofloxacin were nearly 100% from these eye drops, however of dexamethasone were in the range of 93-94%. The recoveries of dexamethasone were consistent and were taken into consideration while calculating for drug content during assay.

2.3.1.3 METHODS FOR THE ASSAY OF BENZALKONIUM CHLORIDE.

Method of assay for benzalkonium chloride from formulations:

EXPERIMENTAL:

The HPLC method reported by Santoni *et al*[244] was standardized to our working conditions. The column used was C_{18}. The mobile phase consisted of a mixture of 30mM tetrabutylammonium hydroxide, adjusted to pH 2.5 with orthophosphoric acid and acetonitrile, in the ratio 30:70 v/v. The flow rate was 1mL/min and the eluent was monitored at 260nm. The injection volume was 20µL. The areas of standard solution of 25µg/mL were used for estimating concentrations of sample solutions. Recoveries of the preservative for the formulations were determined at only one concentration.

RESULTS & DISCUSSION:

Areas of the standard concentration (25µg/mL): 22137, %CV=2.38

Results of the recovery studies of benzalkonium chloride from various formulations listed in Table-8:

TABLE-8: RESULTS OF THE RECOVERY STUDIES OF BENZALKONIUM CHLORIDE FROM VARIOUS FORMULATIONS:

Formulation	Mean % recovery ±s.d. (n=6)
Cip. ophthalmic solution	94.38±3.05
Cip. long-acting gel	93.05±2.55
Cip. ointment	97.58±7.31
Cip. + dexa. suspension	96.92±2.84
Cip. + dexa. solution	94.92±3.49

s.d = standard deviation

The results suggested that the recoveries were above 90% and could be measured with a precision of ±10%. The 10% limit of precision should be acceptable in the case of benzalkonium chloride, since it has very low absorptivity even at 260nm, its wavelength of maximum absorbance. Additionally, very low concentrations need to be injected onto the column, since benzalkonium chloride has a tendency to interact with the column material and may possibly damage the column.

2.3.2 STANDARDIZATION OF DRUGS AND ADDITIVES:

The second part of the preformulation work was to confirm whether the bulk drug/s as well as the additives that go into making a formulation, adhere to their respective pharmacopoeial standards, so as to ensure the quality of the product produced. Furthermore, consistency in quality can be assured only if these are found to comply with the standards laid down for them. The drugs used in the studies were standardized as per their official monographs and the preservative was standardized as well. In case of the other additives, only some of them could be evaluated with a few official tests, that could be conducted in our laboratory. For the rest, the quality control sheets were obtained from their respective manufacturers.

2.3.2.1 CIPROFLOXACIN HYDROCHLORIDE:

The bulk drug sample of ciprofloxacin HCl intended to be used in the delivery systems was standardized by carrying out the tests specified in its official monograph in the USP XXIII.

EXPERIMENTAL:

The various tests that were carried out included: (1) identification by (a) IR spectrum, (b) TLC and (c) Test for chloride; (2) pH of a 1 in 40 solution, (3) Determination of water content, (4) Limit test for sulphate, (5) Limit of fluoroquinolonic acid and (6) Assay.

Although all the tests were carried out as per the official monograph, only the procedure for assay is being briefly described here:

ASSAY:

Mobile phase: A filtered and degassed mixture of 0.025M phosphoric acid, pH adjusted to 3.0±0.1 with triethylamine, and acetonitrile (87:13 v/v).

Standard preparation: 25mg of ciprofloxacin HCl working standard was dissolved in sufficient mobile phase to make 50mL so as to obtain a solution containing 0.5mg/mL of drug.

Resolution solution: A solution containing 0.5mg/mL of analog-A and 0.5mg/mL of ciprofloxacin HCl working standard.

Assay preparation: About 25mg of ciprofloxacin was dissolved in sufficient amount of mobile phase to make 50mL so as to obtain a solution containing 0.5mg/mL of the drug. The liquid chromatograph consisted of a UV detector set at 278nm and a C_{18} column. The mobile phase was pumped at a flow rate of 1mL/min. 20µL of the standard and test preparations were injected on the column and the chromatograms were obtained.

The quantity in, mg, of $C_{17}H_{18}FN_3O_3$.HCl was calculated using the formula: $50C(r_u/r_s)$, in which C is the concentration in mg/mL of the ciprofloxacin HCl working standard in the standard preparation, r_u and r_s are the ciprofloxacin peak areas obtained from the assay preparation and standard preparation, respectively.

Chromatographic purity: The solution for assay preparation was injected as mentioned above, and the percentage of each impurity peak in the chromatogram was obtained by the formula: $100r_i/r_t$, in which r_i is the response of each impurity peak and r_t is the sum of responses of all the peaks.

RESULTS & DISCUSSION:

The results of standardization of ciprofloxacin HCl as per USP are as shown in Table-9:

TABLE-9: RESULTS OF THE STANDARDIZATION OF CIPROFLOXACIN HCl.

TEST	SPECIFICATION	RESULT
1. Identification		
(a) IR Spectrum	To match with standard	Complies
(b) TLC	To match with standard	Complies
(c) Test for Cl^-	Positive	Complies
2. pH of 1 in 40 solution	3.0-4.5	3.97
3. Water	4.7-6.7% w/w	5.4% w/w
4. Sulphate	NMT 0.04%	Complies
5. Limit of fluoro-quinolonic acid	NMT 0.2%	Complies
6. Assay	98.0-102.0% (anhydrous basis)	98.65% w/w
7. Chromatographic purity	Sum of all impurity peaks NMT 0.7%	0.16%
% of analog A	NMT 0.4%	0.09%

The ciprofloxacin HCl sample was found to comply with USP specifications.

2.3.2.2 DEXAMETHASONE:

EXPERIMENTAL:

Dexamethasone bulk drug sample was standardized as per its specifications given in IP 1985. The following pharmacopoeial tests were conducted: (1) Identification by: (a) IR spectrum, (b)

Quantitative colour reaction; (2) Specific optical rotation, (3) Light absorption, (4) Identification of foreign steroids and (5) Assay.

Although all the tests were carried out as per the procedures given in the official monograph, only the procedure for the assay is being briefly described here:

ASSAY:

0.1 gm of each, the test sample and the working standard were weighed and dissolved separately in sufficient aldehyde-free alcohol (AFA) to produce 200mL. 5mL of these were diluted with AFA to 250mL. This gave solutions containing 10µg/mL. 20mL of both these solutions were pipetted out into 50 mL stoppered conical flasks. 20mL of AFA was pipetted out in a third flask, which served as the blank. To each flask, 2.0mL of 0.5% w/v solution of blue tetrazolium in AFA was added and mixed. Subsequently, 2mL of a mixture of 1 volume of tetramethyl ammonium hydroxide (10%) and 9 volumes of AFA was added to each flask and were allowed to stand in dark for exactly 90 minutes at a temperature between 20-35°C.The absorbances of the standard and test solutions were measured at 525nm against the blank. The amount of dexamethasone in the sample was calculated by comparison with the absorbance of the standard solution.

Non pharmacopoeial test:

Determination of the bulk and tap densities of dexamethasone powder: The bulk density of the powder was determined by pouring about 2 gm of dexamethasone into a 10 mL graduated measuring cylinder. The volume occupied by this powder was noted.

Subsequently, the measuring cylinder was tapped several times on a bulk density apparatus and the volume was read again. The bulk and tapped densities were calculated using the formula:

Bulk Density = Weight of powder ÷ Volume occupied

Tapped Density = Weight of powder ÷ Volume occupied after tapping

RESULTS & DISCUSSION:

The results of standardization of Dexamethasone as per IP 1985 are as shown in Table-10. The dexamethasone powder sample was found to comply with IP specifications.

Non-pharmacopoeial test: The bulk density of dexamethasone powder was found to be 1.33 g/mL and the tapped density was found to be 1.467 g/mL. These are important parameters for the preparation of suspensions, since some of the properties of suspensions like the sedimentation rate will depend partly on the density of the suspended powder.

2.3.2.3 BENZALKONIUM CHLORIDE:

EXPERIMENTAL:

Benzalkonium chloride 50% w/v solution was analyzed as per the tests given in its official monograph in the Indian Pharmacopoeia 1985. The following tests were carried out: (1) Identification by: (a) Reaction with nitric acid and $HgCl_2$ solution, (b) Colour reaction with β-naphthol, (c) Reaction to litmus and foaming ability, (d) Presence of chlorides; (2) Presence of ammonia compounds, (3) Presence of foreign amines, (4) Content of alcohol by gas chromatography and (5) Assay.

TABLE-10: RESULTS OF THE STANDARDIZATION OF DEXAMETHASONE:

TEST	SPECIFICATION	RESULT
1. Identification		
(a) IR Spectrum	To match with standard	Complies
(b) TLC	To match with standard	Complies
(c) Extinction of coloured complex at 423nm	NLT 250	269
2. Specific optical rotation	+ 72' to + 80'	+77.77'
3. Light absorption at 240 nm	0.38-0.41	0.394
Ratio at 240 & 263 nm	1.9-2.1	2.02
4. Related foreign steroids	To comply	Complies
5. Loss on drying	NMT 0.5% w/w	0.27% w/w
6. Assay	96-104%	98.65%w/w

Although all the tests for analysis of benzalkonium chloride were carried out as per the procedures given in the official monograph, only the procedure for assay is being briefly described here:

ASSAY:

25mL of a 4% w/v aqueous solution of benzalkonium chloride was transferred to a separating funnel. To this, 25mL of chloroform, 10mL of 0.1N NaOH and 10mL of freshly prepared 5% w/v solution of KI were added. The mixture was shaken vigorously and the

chloroform layer was discarded. The aqueous layer was washed thrice with 10mL quantities of chloroform and the chloroform layers were discarded. To this solution, 40mL of concentrated HCl was added, cooled and titrated with a 0.05M solution of KIO_3 till the end point was reached. A blank titration was also carried out. The difference between the two titrations represented the amount of KIO_3 used by benzalkonium chloride.

Each mL of 0.05M KIO_3 ≡ 0.0354 gm of $C_{22}H_{40}ClN$.
The amount of $C_{22}H_{40}ClN$ weight in volume was calculated using the specific gravity of the solution.

RESULTS & DISCUSSION:

The results of standardization of benzalkonium chloride as per IP 1985 are as shown in Table-11. The above results suggest that the sample of benzalkonium chloride complied with the IP specifications.

2.3.2.4 OTHER ADDITIVES:

EXPERIMENTAL:

A few of the pharmacopoeial tests were carried out on the samples of HPMC E4M, Tween 80 and Poloxamer-407. The various tests carried out are as follows:carried out were as follows:

TABLE-11: RESULTS OF THE STANDARDIZATION OF BENZALKONIUM CHLORIDE

TEST	SPECIFICATION	RESULT
1. Identification		
(A) Reaction with nitric acid and $HgCl_2$ solution	Precipitate forms which is insoluble in alcohol	Complies
(B) Colour reaction with β-naphthol	Formation of an orange-red coloured complex	Complies
(C) Reaction to litmus and foaming ability	Neutral or slightly alkaline, foams strongly	Complies
(D) Presence of Cl-	To comply	Complies
2. Ammonia compounds	To be absent	Absent
3. Foreign amines	To be absent	Absent
4. Alcohol	NMT 16%v/v	4.8%v/v
5. Assay	49-51%w/v	49.88%w/w

2.3.2.4 contd...

(A) HYDROXYPROPYL METHYLCELLULOSE 2910 NF (HPMC E4M PREMIUM):

The various tests carried out on HPMC 2910 included:

1. IDENTIFICATION:

(a) About 1 gm of the powder was gently added on the surface of 100mL of distilled water in a beaker and was allowed to disperse over the surface. The beaker was allowed to stand till the substance became transparent and mucilaginous. The contents were mixed with the help of a magnetic stirring bar. An equal volume of either 1N HCl or 1N NaOH was added.

(b) About 1 gm was added to 100mL of boiling distilled water and the mixture was stirred. The slurry was cooled to 20°C, the characteristics of the resulting solution were observed.

(c) A few mL of the solution prepared in 'Identification b' was poured onto a glass plate and the water was allowed to evaporate.

2. APPARENT VISCOSITY:

The viscosity of a 2% w/w solution of the polymer was determined using a Brookefield viscometer at 20°C using spindle no. S-18.

3. LOSS ON DRYING:

About 1 gm of the powder was heated at 105°C to constant weight.

(B) POLOXAMER 407 (NF):

Only one test could be conducted on the sample of poloxamer, obtained from BASF, Germany.

The pH of a 1 in 40 solution was determined using a pH meter.

(C) TWEEN 80 (POLYSORBATE 80 NF):

The following pharmacopoeial tests were carried out on Tween 80:

1. SPECIFIC GRAVITY:

The test sample was filled into a 5mL capacity tared specific gravity bottle at 25°C and the weight of the sample filled in was measured on an analytical balance.

2. WATER CONTENT:

The amount of water in the sample was determined by the Karl Fischer method, 0.5 gm of sample was used.

RESULTS & DISCUSSION:

The results of the analysis of HPMC E4M, Poloxamer-407 and Tween 80 are as shown in Table-12:

TABLE-12: RESULTS OF THE STANDARDIZATION OF OTHER ADDITIVES

ADDITIVE	TEST	SPECIFICATIONS	RESULT
HPMC E4M	Identification a	No change in solution	Complies
	Identification b	Clear mucilaginous solution	Complies
	Identification c	Formation of a self-sustaining film	Complies
	Viscosity	3000-5600 cps	3800cps
	LOD	NMT 5.0% w/w	3.78%
POLOXAMER 407	pH of solution	5.0 - 7.5	6.14
TWEEN 80	Specific gravity	1.06-1.09	1.08
	Water content	NMT 3.0% w/w	1.72%

It was found that the other additives, complied with their respective pharmacopoeial specifications.

2.3.3 DRUG-ADDITIVE COMPATIBILITY STUDIES, SELECTION OF SUITABLE CONTAINER, SELECTION OF ESSENTIAL ADDITIVES AND ESTABLISHING METHODS OF STERILIZATION OF DRUG POWDERS:

The third part of the preformulation studies was aimed at selection of suitable additives, preservative-s, container etc., to be used in preparation of the product. Before that it is essential to first study their physical and chemical compatibility with the drug. Since a majority of the ophthalmic

dosage forms are aqueous in nature, the solution state compatibility studies are of prime importance. Solution state compatibility is assessed by mixing together aqueous solutions of the drugs and additives, such as, tonicity adjusting agents, preservatives, stabilizers, etc. and keeping them at accelerated conditions of temperature so as to enhance the rate of reaction, if any were to occur. After the prescribed period of storage (minimum of 3 weeks), the solutions are observed for visual changes such as change in colour, clouding or precipitation, as well as for chemical changes like drug content, purity etc.

Dry powders are sterilized by either exposing them to dry heat, gamma-irradiation or ethylene oxide gas. The first method is referred to as 'hot method', as heat energy is used to sterilize the powder. The latter two methods are referred to as cold methods since no heat energy is involved. Here, the sterilization takes place because of ionizing radiation or due to chemical modifications on the microbial surface. The hot method is reserved for those powders or solids that have the ability to retain their physicochemical integrity after exposure to heat. However, not all powders or solids can withstand this drastic treatment and may undergo physicochemical changes, such as, loss of water of crystallization, charring, change in colour due to oxidation, polymerization, change in polymorphism, etc. Such powders or solids are thermolabile in nature and have to be sterilized by the cold methods. Again, all drugs in their solid form may not be stable on being irradiated with gamma-radiation and certain compounds may react with the highly reactive EtO. The

method of sterilizing a drug substance has to be decided at the preformulation stage.

Methods of sterilization of ciprofloxacin HCl and dexamethasone were thus established.

2.3.3.1 COMPATIBILITY STUDIES OF CIPROFLOXACIN HCl WITH VARIOUS ADDITIVES:

EXPERIMENTAL:

The compatibility study of ciprofloxacin HCl with various additives was carried out by mixing together aqueous solutions of the two. These solutions were then analyzed for drug content, by HPLC, and observed for physical and chemical changes, after storage for a month at 5°C, 25°C and 45°C. A 1.4% w/v solution of ciprofloxacin HCl (equivalent to 1.2% w/v of ciprofloxacin base) was mixed with each of the solutions of the additives, listed below, in such a manner that the final solution contained 0.35% w/v of ciprofloxacin HCl and the following concentrations of additives:

(a) tonicity modifiers: sodium chloride (0.9%w/v), mannitol (5.0% w/v), propylene glycol (2.0% w/v), glycerol (2.5% w/v), dextrose (5.0% w/v) and boric acid (1.9% w/v).

(b) preservatives: benzalkonium chloride (0.01% w/v) and thiomersal (0.01% w/v).

(c) Stabilizers: sodium metabisulfite (0.02% w/v) and disodium EDTA (0.1 w/v).

(d) viscosity-imparting agents: HPMC E4M (0.1% w/v), Carbopol 940 (0.1% w/v), Carbopol 971 (0.1% w/v) poloxamer 407 (20% w/w).

Amongst the drugs, 0.1% w/v solution of dexamethasone sodium

phosphate was evaluated for compatibility with ciprofloxacin HCl.

RESULTS & DISCUSSION:

(A) Tonicity adjusting agents: Ciprofloxacin HCl (CipHCl) in solution was found to be physically as well as chemically compatible with all the tonicity adjusting agents, namely, sodium chloride, mannitol, propylene glycol, glycerol, dextrose as well as boric acid. The solutions on being stored at 45°C did not show loss of drug, or any discolouration. No physical changes, such as, precipitation, cloudiness or crystallization were observed. The pH of the solutions too did not change appreciably. The UV spectra of ciprofloxacin in the presence of these additives was superimposible with that of ciprofloxacin itself.

Mannitol, propylene glycol, glycerol, boric acid and dextrose are all non-ionic in nature, and hence the possibility of a chemical reaction with ciprofloxacin HCl was quite less. These non-ionic compounds may however cause problems of solubility of ciprofloxacin HCl since they are used at reasonably high concentrations and may thus cause ciprofloxacin HCl to precipitate or crystallize out. On the other hand, sodium chloride ionizes in aqueous solutions and forms Cl^-, an ion common to both, sodium chloride as well as CipHCl. Thus, due to the common ion effect, sodium chloride can suppress the ionization of ciprofloxacin, which would also lead to a decrease in its solubility. This fact becomes more relevant while deciding the order of addition of components while preparing the formulation. Due to this phenomenon taking place, sodium chloride

solution should be added after dissolving CipHCl.

TABLE-13: RECOVERY STUDIES OF CIPROFLOXACIN IN THE PRESENCE OF VARIOUS ADDITIVES AND THE pH OF THE RESULTING SOLUTIONS:

Tonicity adjusting agent	Temperature	Drug content ± s.d.	pH
Sodium chloride	5°C 25°C 45°C	99.69±0.39 100.34±0.49 99.36±0.46	4.45 4.45 4.47
Boric acid	5°C 25°C 45°C	99.90±0.62 99.95±0.46 100.23±0.78	4.45 4.44 4.44
Propylene glycol	5°C 25°C 45°C	99.81±0.60 99.21±0.79 99.84±0.46	4.51 4.50 4.53
Glycerol	5°C 25°C 45°C	99.29±0.63 100.03±0.77 99.55±0.77	4.45 4.47 4.44
Dextrose	5°C 25°C 45°C	100.38±1.00 100.02±0.88 100.12±0.96	4.48 4.48 4.47
Mannitol	5°C 25°C 45°C	99.96±0.77 99.25±0.40 100.32±0.98	4.50 4.52 4.50

Table-13 shows that CipHCl that had been mixed with the solutions of different tonicity adjusting agents, could be recovered completely, which shows lack of any chemical interaction between them and CipHCl.

(B) Preservatives: Among the preservatives, benzalkonium chloride (BKC) was found to be physically as well as chemically compatible with CipHCl solution. The mean ciprofloxacin content was found to be 100.20±0.85 (n=3) and the pH of the solution was 4.52. BKC is a quaternary ammonium compound and is hence cationic in nature, and CipHCl in aqueous solution is also in the form of a cationic

species, namely the ciprofloxacinium ion. Since both, the drug as well as the preservative are present in the cationic form, there should ideally be no possibility of any interaction. Even though BKC and CipHCl possess a common chloride ion, the solubility of neither should be affected since the concentration of BKC is very low.

Thiomersal was found to be incompatible with CipHCl solution at 45°C. There was formation of a sticky precipitate on the sides of the vial. This incompatibility was not immediately observed. The precipitate formed after about 7 days of storage at 45°C and was not seen in solutions stored at 25°C and 5°C even after a month. Thiomersal is a mercurial preservative and undergoes degradation in aqueous solution to generate Hg^{+2}, which too possesses antimicrobial activity. However, this is a slow reaction and may take months for complete degradation to occur, and this reaction can accelerated by heat. This reaction could be probably due to the interaction of Hg^{+2} ions, generated by thiomersal and chloride ions generated by CipHCl, in aqueous solution, leading to formation of $HgCl_2$ and causing precipitation of ciprofloxacin base. In order to confirm this phenomenon, 50mL of 0.35 %w/v solution of ciprofloxacin was boiled after adding 10mg of thiomersal, a sticky precipitate was formed after about 10 min, thus proving the incompatibility.

(C) Stabilizers: Among the stabilizers evaluated, sodium metabisulphite was found to be incompatible with CipHCl solution. The solution developed a distinct yellow-orange colour within 24 hours of storage at 25°C. On analyzing the solution for purity of

ciprofloxacin, it was found that the content of Analog-A, a degradation product of ciprofloxacin had increased from 0.09% in the bulk drug to 2%. No attempt was made to elucidate the mechanism of degradation. Since there was an incompatibility, it was decided not to use sodium metabisulphite in the formulations. Disodium EDTA at 0.1% w/v concentration was found to be physically as well as chemically compatible with CipHCl solution when stored at 5°, 25° and 45°C. The drug content at these temperatures after 1 month was 100.12±0.66, 99.10±0.86 and 99.99±0.55. The pH of these solutions were 4.48, 4.50, 4.45, respectively

(D) **Viscosity-imparting agents:** Among these, HPMC E4M and Poloxamer-407 were found to be chemically as well as physically compatible with CipHCl solution. The content and purity of CipHCl was unchanged in these solutions. The results of the recovery of CipHCl from the mixture with different polymer solutions are listed in Table-14:

TABLE-14: RESULTS OF THE RECOVERY STUDIES OF CIPROFLOXACIN IN THE PRESENCE OF VARIOUS POLYMERS AND THE pH OF THE RESULTING SOLUTIONS:

Polymer	Temperature	Mean drug content (n=3)	pH
HPMC E4M	5°C 25°C 45°C	99.68±0.55 99.20±0.70 100.22±0.40	4.51
Poloxamer-407	5°C 25°C 45°C	100.28±0.42 99.55±0.70 99.37±1.02	4.48

The lack of any interaction between CipHCl and these polymers may be attributed to the non-ionic and inert nature of the polymers. The UV spectra of CipHCl in the presence of these polymers was superimposible with that of CipHCl alone, indicating lack of any interaction. On the other hand Carbopol^{R}, which is an acrylic acid polymer was found to be incompatible with CipHCl solution. On adding CipHCl solution to a slurry of Carbopol^{R}, a copious off-white precipitate was formed instantaneously. This precipitate however, dissolved with addition of excess alkali. Carbopol^{R} is a polyacrylic acid polymer and is only partially ionized in plain water. On addition of alkali, it slowly undergoes hydration and ionization to the anionic form, which probably was incompatible with the cationic CipHCl and resulted in formation of an insoluble complex.

At alkaline pH, the acidic polymer is completely ionized and ciprofloxacin being zwitterionic, too solubilizes due to formation of its sodium salt. However, the polymer loses its viscosity at this pH and the solubilization is thus of no value. Therefore Carbopol^{R} was not used in the preparation of the gels.

Amongst the anti-inflammatory drugs, dexamethasone sodium phosphate was chosen, since eye drops containing a combination of the two were also to be prepared. On mixing together aqueous solutions of the two, immediate precipitation occurred. This could be due to the alkaline nature of dexamethasone sodium phosphate which caused precipitation of ciprofloxacin. Hence, for the combination eye-drops, dexamethasone which is non-ionic in

nature was used.

2.3.3.2 SELECTION OF CONTAINER FOR DISPENSING CIPROFLOXACIN HCl SOLUTION:

Containers are an integral part of any product. They are not only used to dispense the formulation, but also serve to preserve the sterility of the contents and also protect the contents from being contaminated by environmental factors such as light, oxygen, etc. Type I and type II glass vials are used to dispense parenteral solid as well as liquid preparations. Colourless glass is permeable to UV as well as visible radiation, hence only those products that are photostable can be dispensed in a container made from this type of glass. However, photosensitive drugs need to be dispensed in amber coloured glass vials. The amber colour is imparted by ferric oxide this makes the glass impermeable to long UV radiation. Some amount of visible radiation can still transmit through it, but it is the UV light that is more destructive.

EXPERIMENTAL:

To decide on the type of vial needed to dispense the formulations, 0.35%w/v CipHCl solution was filled in type I amber and colourless vials and exposed to direct sunlight for a period of 1 month (from 6.5.94 to 6.6.94). After a month, the solutions were evaluated for visual changes in colour and chemical purity was measured using HPLC. In order to check visually for any change in colour, the solutions were emptied into Nessler's cylinder.

RESULTS & DISCUSSION:

It was found that the solution in the colourless vial had acquired a distinct yellow-orange colour. The solution stored in amber coloured vial however remained colourless. From this study it was evident that prolonged exposure to light does cause discolouration of CipHCl and that degradation can be avoided by dispensing the solutions in amber coloured vial, which cuts-off the short as well as long UV light. The two solutions were analyzed for drug purity and content by HPLC. It was found that the amount of Analog-A in the discoloured solution had gone upto 2%. However, the content of Analog-A in the solution stored in amber vial remained unchanged from the original solution.

Thus it was decided to dispense CipHCl solutions in amber coloured vials.

2.3.3.3 TO ASSESS THE NEED OF INCORPORATING A CHELATING AGENT IF THE FORMULATION NEEDS TO BE DISPENSED IN AN AMBER COLOURED VIAL:

Since Fe^{+3} may leach out of the amber coloured vials and may interact with CipHCl, the feasibility of incorporating a chelating agent, that would competitively bind to Fe^{+3} was explored.

EXPERIMENTAL:

Amber vials were filled with 0.35% w/v solution of CipHCl with and without 0.1% w/v disodium EDTA, a chelating agent. In order to accelerate the leaching of Fe^{+3}, the vials were autoclaved for 20 minutes, at 121°C and 15 psi pressure. After removing from the autoclave, the solutions were allowed to cool and were

emptied out into Nessler's cylinders to check for change in colour. Additionally, the absorbances of the undiluted solutions were measured at 420nm using a double beam spectrophotometer and using freshly prepared 0.35% w/v solution of CipHCl and that additionally containing 0.1% w/v EDTA as well, as the respective blanks. Any increase in the absorption in the above mentioned range would indicate leaching of Fe^{+3} into the solution.

RESULTS & DISCUSSION:

The two CipHCl solutions, with and without EDTA when viewed in a Nessler's cylinder revealed no perceptible difference. However, when the absorbances of these solutions were measured at 420nm, the solution without EDTA was found to have increased absorbance. The absorbance value of this solution as compared to the blank was 0.011 absorption units, whereas that of the EDTA solution, with 0.1% w/v EDTA as the blank was only 0.001 absorption units. This probably means that EDTA prevented the increase in absorbance of CipHCl solution.

Amber glass contains ferric oxide as tinting agent. The ferric oxide is soluble in acidic solutions and may thus leach out of the glass. CipHCl is known to chelate metal ions like Ca, Al, Mg as well as Fe which results in the formation of large molecular complexes, which are devoid of antibacterial activity and are unabsorbable by the body tissues. A solution of CipHCl is acidic in nature and thus there are chances that it may lead to leaching out of ferric ions. Though the leaching cannot be prevented, the

interaction of Fe^{+3} with CipHCl can be prevented by incorporating a competitive chelating agent, such as EDTA, which is widely used in pharmaceutical preparations, for this purpose.

2.3.3.4 METHOD OF STERILIZING CIPROFLOXACIN HCl AND DEXAMETHASONE POWDERS:

EXPERIMENTAL:

Since ciprofloxacin HCl is a monohydrated compound, it could not be sterilized by dry heat. Therefore, the feasibility of sterilizing it by ethylene oxide gas was explored. For this purpose, micronized CipHCl powder was filled in thin polyethylene bags, such that on spreading it evenly in the bag, the layer of powder was not more than a few millimeters thick. These were sterilized with 100% ethylene oxide by using the following sterilization cycle:

(a) 2 steam pulses: 11 minutes each

(b) Humidification: 98 minutes

(c) Gas pressure: 1.28kg/m^3

(d) Gas exposure time: 361 minutes

(e) Nitrogen purging: 2 pulses, 40 minutes each

After exposure to EtO, CipHCl powder was evaluated for possible physicochemical changes like: drug content by HPLC, changes in molecular structure using UV/IR spectroscopy and HPLC. The powder was also assessed for sterility, by the membrane filtration method.

Dexamethasone IP was subjected to dry heat sterilization. Sterilization was attempted at 160°C and 140°C for 2 and 3 hours, respectively, in hot air oven and *in vacuo*. After heating, the

powders were evaluated for changes in percentage purity, melting profile and molecular structure. The powder was also assessed for sterility by the direct inoculation technique.

RESULTS & DISCUSSION:

The results which are summarized in Table-15, show that there was no change in the CipHCl powder after exposing it to EtO (Figs. 5 and 6). Thus EtO can be used to sterilize CipHCl powders.

Since CipHCl needs to be sterilized by the "cold methods", the choice was between gamma-radiation and EtO sterilization. Since radiation is reported to cause colour changes, we studied the feasibility of EtO sterilization.

TABLE-15: RESULTS OF THE EVALUATION OF STERILIZING CIPHCl BY EXPOSURE TO EtO GAS:

METHOD	PARAMETER	ORIGINAL VALUE /DESCRIPTION	AFTER EXPOSURE TO ETO GAS
VISUAL OBSERVATION	Appearance	Yellowish crystalline powder	
HPLC	Drug content	98.65%	98.63%
	Retention time	5.792 min	5.775 min
	% purity	99.82	99.77
IR SPECTRUM	Changes in characteristic peaks	Both the spectra were found to be superimposible	
STERILITY TEST	Sterility	___	Sterile

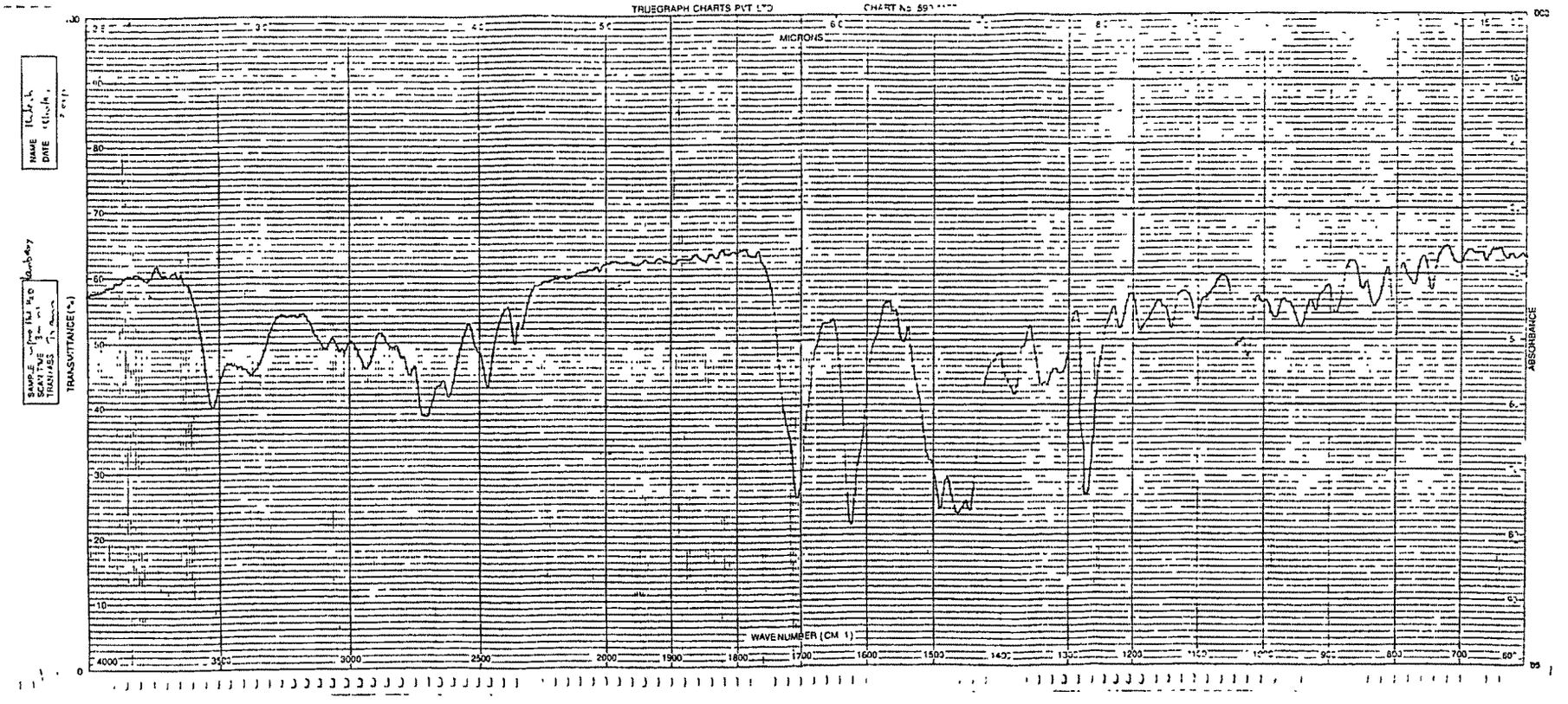

Fig. 5a: Infra-red spectrum of Ciprofloxacin hydrochloride USP

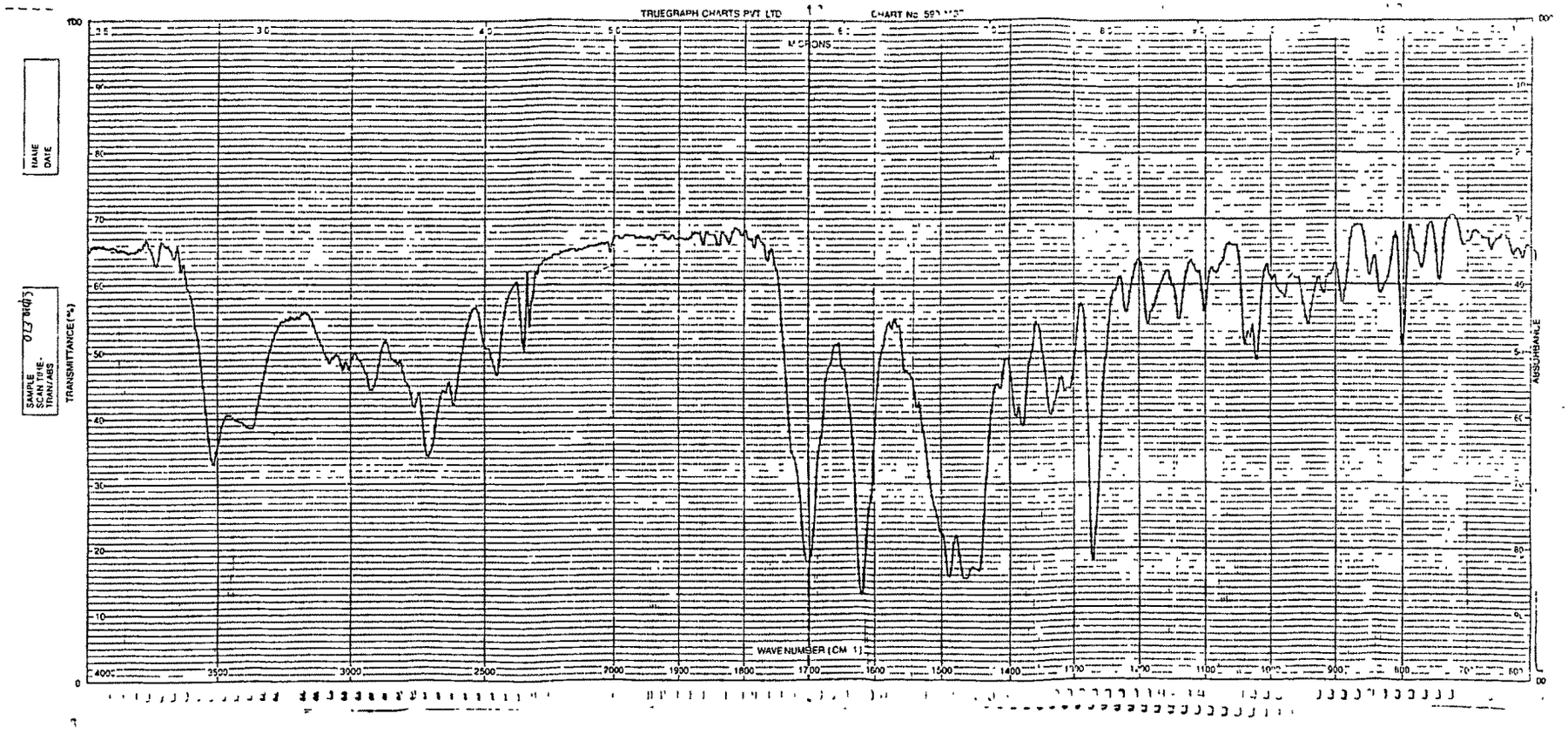

Fig. 5b: Infra-red spectrum of Ciprofloxacin hydrochloride USP sterilized by ethylene oxide

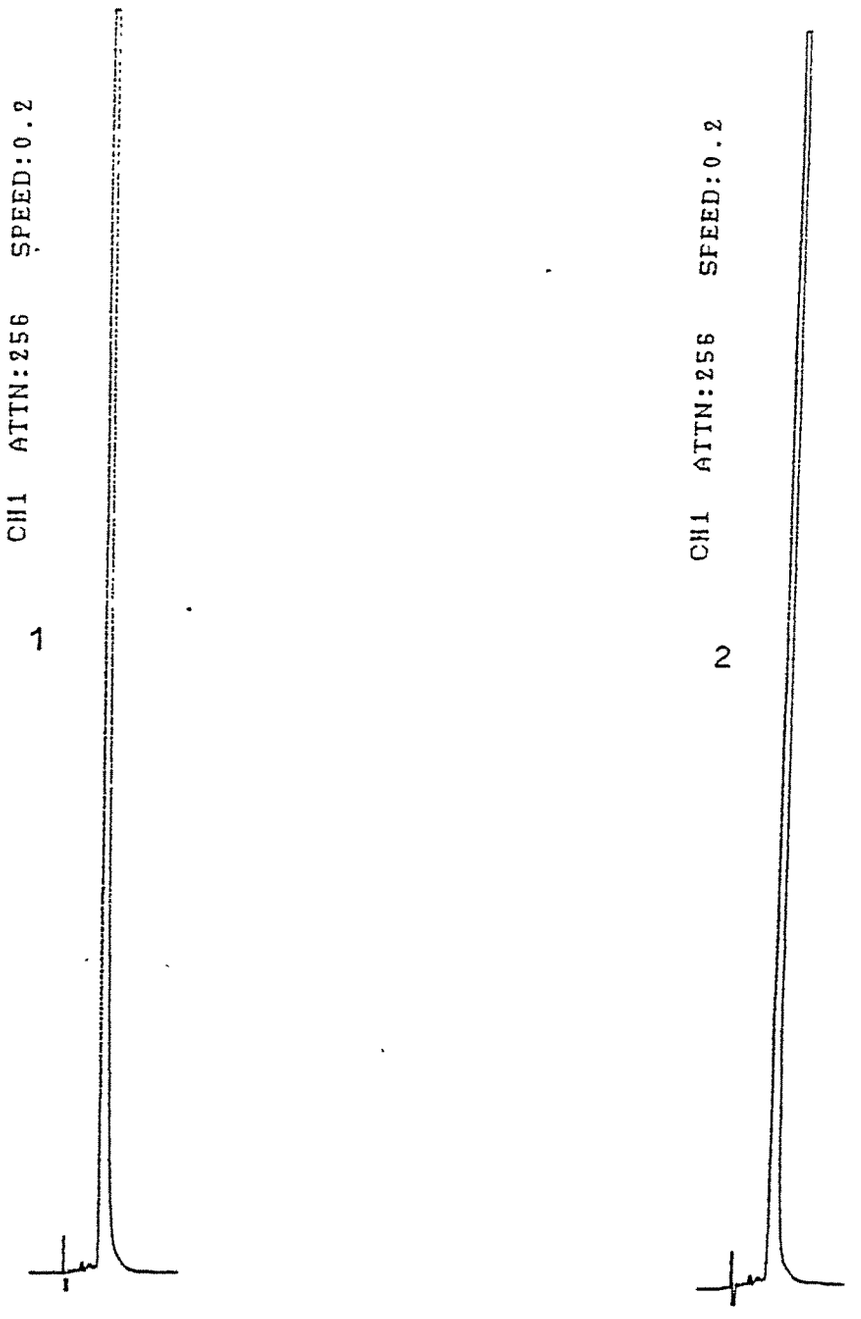

Fig. 6a: Chromatogram of (1) Ciprofloxacin HCl USP and (2) Ciprofloxacin HCl USP sterilized by ethylene oxide

(B) Dexamethasone powder when heated at 140°C and 160°C for a period of 3 and 2 hours, respectively in a hot air oven, resulted in discolouration of the drug. Heating the powder at the same temperature *in vacuo* helped in decreasing the discolouration. The discolouration was reduced at 160°C and was totally absent at 140°C. The powders thus sterilized were analyzed for purity by HPLC and it was found that all those heated in the hot air oven, underwent significant degradation and one of the characteristic peaks in the IR spectrum too showed splitting (Fig. 7). However, degradation was totally absent when dexamethasone was sterilized at 140°C *in vacuo* and the IR spectrum matched with that of the unsterilized drug (Table-16).

TABLE-16: RESULTS OF THE EVALUATION OF STERILIZING DEXAMETHASONE AT 140°C *IN VACUO* AND IN AIR:

METHOD	PARAMETER	ORIGINAL VALUE/ DESCRIPTION	AFTER EXPOSURE TO HEAT AT 140°C FOR 3h *IN VACUO*	AT 140°C FOR 3h IN AIR
VISUAL OBSERVATION	Appearance	White crystalline powder		Off white powder
HPLC	Drug content	98.65%	98.67%	---
	Retention time	8.058min	8.067min	2 peaks
	% purity	100.00	100.00	87.38
IR SPECTRUM	Changes in peaks	Both the spectra were found to be superimposible		Peak splits observed
DSC	Melting temp.	277.41°C	277.22°C	~280°C
STERILITY TEST	Sterility	---	Sterile	---

The change in colour of dexamethasone on heating in a hot air oven could be attributed to the oxidation of the dihydroxy acetone side-chain attached to C_{17} of the steroid nucleus. This would probably lead to the formation of more non-polar compounds. The chromatograms of samples heated in the hot air oven at 140°, 160° and *in vacuo* at 160°C showed an additional peak well after dexamethasone (Fig. 8). Since the additional peak eluted out later than dexamethasone on a reversed phase column, it supports the hypothesis of formation of relatively non-polar compounds.

However, no additional peak was observed in the chromatogram of dexamethasone sterilized at 140°C (Fig. 8) *in vacuo* which indicates that at this temperature, sterilization was possible without causing any harm to the stability of the drug. The DSC scan (Fig. 9) of standard dexamethasone and that sterilized *in vacuo* were found to be superimposible. However the melting endotherm of the sample sterilised in air was expanded indicating that changes had occurred.

Additionally, this powder on being checked for sterility by the direct inoculation method was found to be sterile.

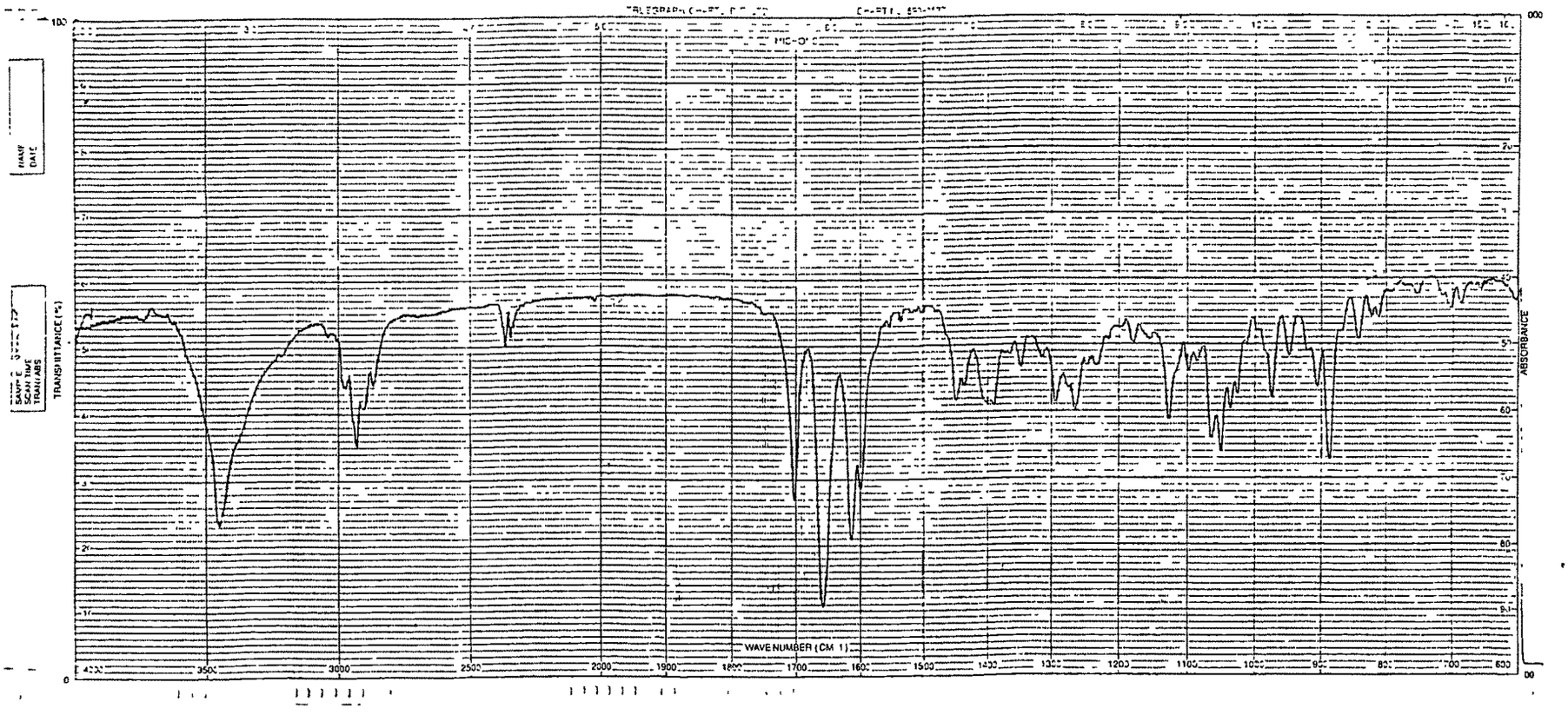

Fig. 7a: Infra-red spectrum of Dexamethasone IP

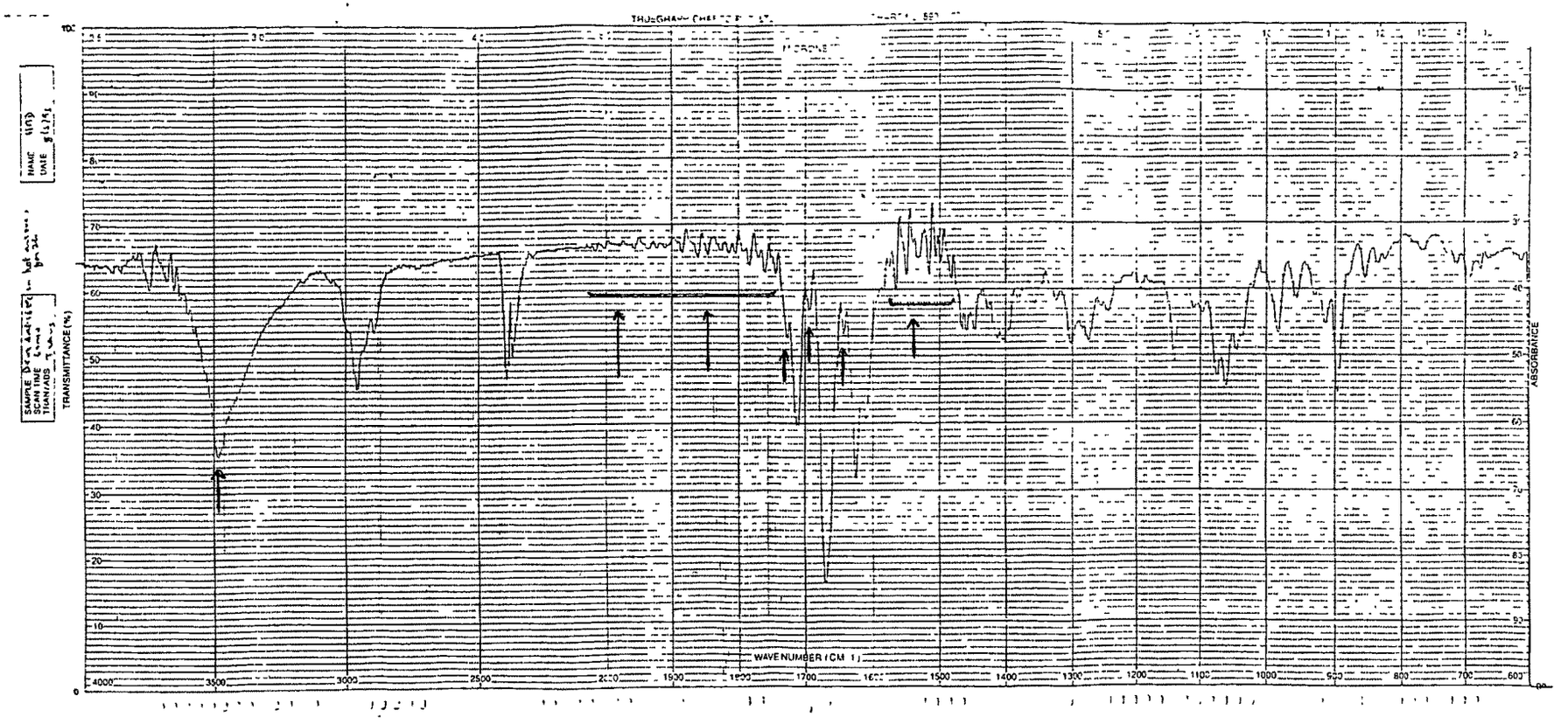

Fig. 7b: Infra-red spectrum of Dexamethasone IP sterilized by heating at 140°C in hot air oven
(Peak changes are indicated by arrow marks)

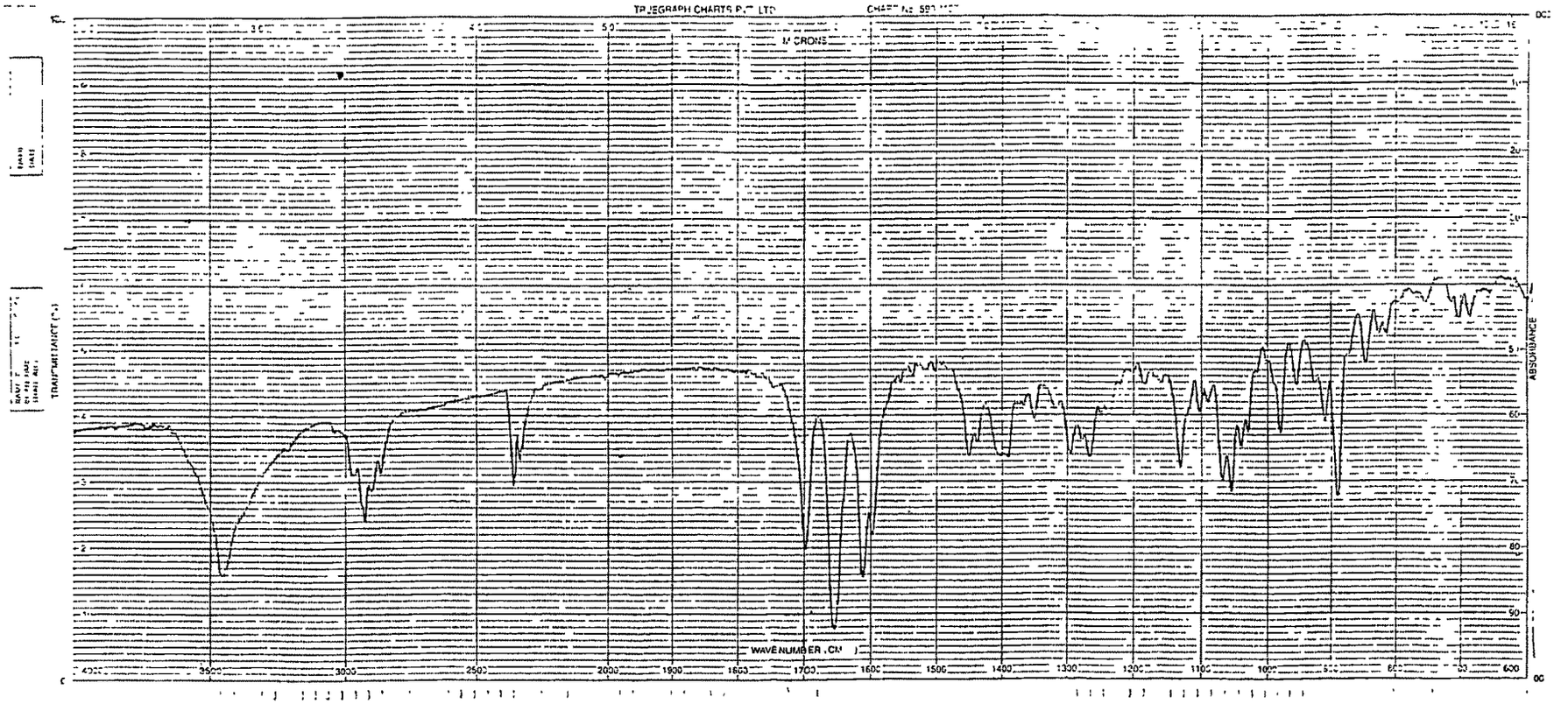

Fig. 7c: Infra-red spectrum of Dexamethasone IP sterilized by heating at 140°C *in vacuo*

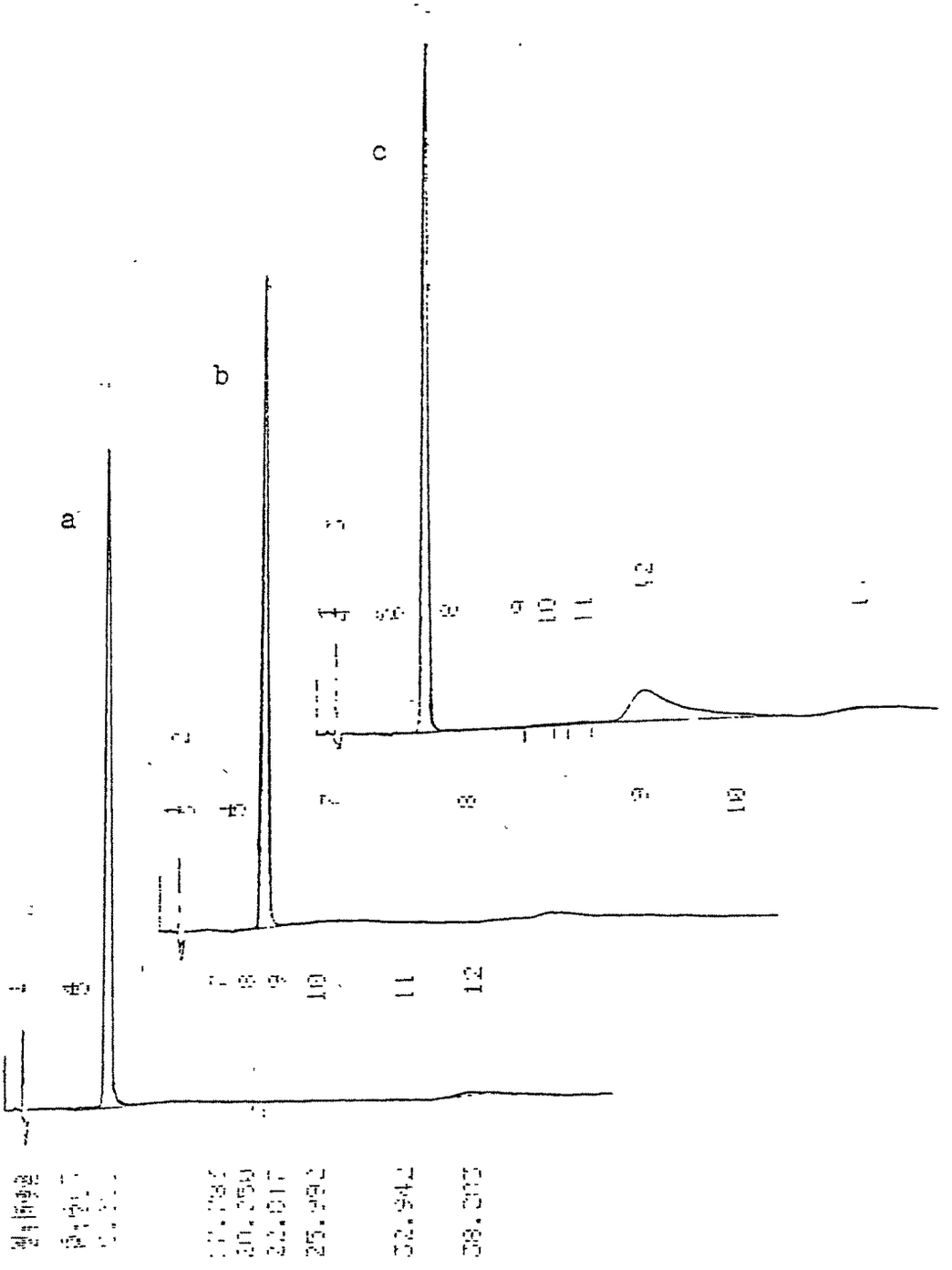

Fig. 8: Chromatograms of (a) Dexamethasone IP, (b) Dexamethasone IP sterilized by heating at 140°C *in vacuo* and (c) Dexamethasone IP sterilized by heating at 140°C in hot air oven

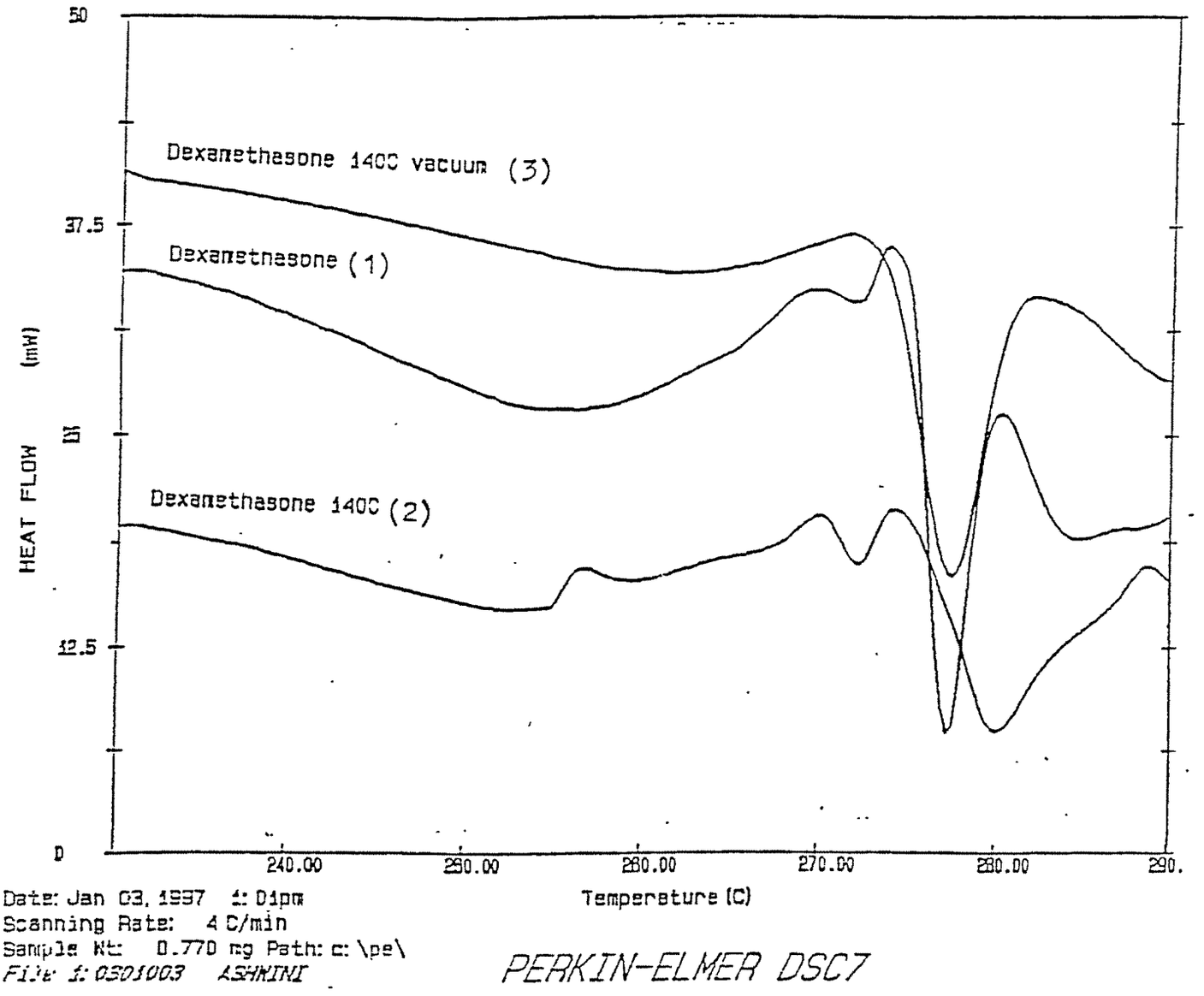

Fig. 9: DSC scan of (1) Dexamethasone IP, (2) Dexamethasone IP sterilized by heating at 140°C in hot air oven and (3) Dexamethasone IP sterilized by heating at 140°C *in vacuo*

2.3.3.5 METHOD OF STERILIZATION OF CIPROFLOXACIN SOLUTION:

The CipHCl solution had to be sterilized prior to being dispensed. Terminal sterilization by autoclaving is a popular means of sterilizing ophthalmic solutions. However, not all solutions can withstand autoclaving. Some solutions may not necessarily undergo chemical degradation but may also show signs physical instability in terms of crystallization of the dissolved drug/additive or shedding of glass particles by the container. Hence prior to preparing the formulation, it is essential to establish a suitable method of sterilization.

EXPERIMENTAL:

A 0.35%w/v CipHCl solution was rendered isotonic with either NaCl, propylene glycol, glycerol, mannitol or boric acid, which were the proposed tonicity adjusting agents. These solutions were either filled in amber coloured vials and autoclaved or were membrane filtered and filled into sterile amber coloured vials. The filled vials were stored at 5°C and 45°C for a period of 1 month and evaluated for drug content and visible physical changes like precipitation/crystallization or change in colour. Sterility of the solutions was checked immediately after filling and sealing, by the USP membrane filtration method.

RESULTS & DISCUSSION:

Autoclaving the drug solution did not cause it to degrade, as can be seen from the results of drug assay in Table-17. However, isotonic CipHCl solutions that were sterilized by autoclaving

developed needle shaped crystals on storage overnight at room temperature, 5°C as well as 45°C. On the other hand, solutions that were sterilized by passing through membrane filter were absolutely clear and did not show formation of crystals either at 5°C or 45°C, even after a month. The solution, thus filter sterilized was found to be sterile.

TABLE-17: RESULTS OF THE ASSAY AND MEASUREMENT OF pH OF CIPHCl SOLUTION, BEFORE AND AFTER STERILIZATION:

	DRUG CONTENT	pH
CONTROL SOLUTION	99.17±0.68	4.46
AFTER AUTOCLAVING	99.59±0.59	4.49
AFTER MEMBRANE FILTRATION	99.68±0.77	4.45

Although type-1 glass is neutral borosilicate glass, it does contain about 4% of sodium oxide which is alkaline in nature and may easily leach out in the filled solution on autoclaving. This leaching, however minuscule, may lead to minor changes in pH and cause the drug to precipitate or crystallize out. On the other hand if autoclaving is omitted, this leaching process can be greatly minimized. Therefore, it was decided to use the membrane filtration method for sterilizing solutions of CipHCl. Additionally, it was decided to incorporate a weak buffering agent (acetate buffer) in the solution so as to prevent changes in pH.

2.4 PREPARATION OF DOSAGE FORMS:

After conduct of necessary preformulation investigations, the following preparations were prepared using standardized materials and methods with only the essential additives. The preparations were then evaluated for essential product qualities.

i) 0.3% w/v ciprofloxacin ophthalmic solution ***(Cip solution)***
ii) 0.3% w/w ciprofloxacin ophthalmic gel(long-acting) ***(Cip gel)***
iii) 0.3% w/w ciprofloxacin ophthalmic ointment ***(Cip ointment)***
iv) 0.3% w/v ciprofloxacin and 0.1% w/v dexamethasone ophthalmic solution ***(Cip+Dexa solution)***
v) 0.3% w/v ciprofloxacin and 0.1% w/v dexamethasone ophthalmic suspension ***(Cip+Dexa suspension)***

The procedure for preparation of dosage forms was divided into two parts, namely, (i) cleaning and sterilization of the containers/closures and (ii) preparation of the dosage forms.

2.4.1 CLEANING AND STERILIZATION OF CONTAINERS/CLOSURES:

In the process of preparation of dosage forms, the pharmacist has to be careful about the quality of the container/closure that will be used to dispense the formulation. Even type-1 glass contains alkaline oxides, upto a certain extent and hence new glassware should first be immersed in acid solutions so as to neutralize the surface alkalinity. This alkalinity if not neutralized, can cause changes in pH of the formulation filled in that container and may lead to instability. Similarly, the glass or the closures can leach out other chemicals or may contribute to particulate matter and render the product unfit for use. The rubber closures are known to leach out a number of organic chemicals, especially plasticizers. Stoppers made of low density

polyethylene (LDPE) are more or less inert in nature and are therefore gaining popularity. Their major drawback is that they melt on autoclaving.

It is therefore necessary to clean and sterilize the containers and closures before using them to dispense the formulations.

EXPERIMENTAL:

Before using the vials, they were subjected to the powdered glass test for alkalinity, which is the official test for type-1 glass in the USP. Type-1 glass amber vials that were used for dispensing the solution were cleaned and sterilized as follows:

(i) The vials were rinsed with tap water and soaked in 5% v/v nitric acid, for a period of about 10 hours, so as to neutralize the surface alkalinity.

(ii) The vials were then rinsed with tap water and then immersed in 0.5% w/v Teepol^{R} solution for a period of 2 hours. The vials were then scrubbed with a soft brush and rinsed with water.

(iii) The vials were again soaked in 5% v/v nitric acid for 30 minutes so as to remove excess soap from the surface of the vials.

(iv) The vials were rinsed with tap water, distilled water and finally rinsed with sterile membrane filtered double distilled water in the laminar flow hood.

(v) The vials were placed inverted in an enameled tray, covered with an aluminium foil transferred to a hot air oven for sterilization. The vials were sterilized at 160°C for a period of 3 hours.

One day before filling the sterile drug solutions, three vials were sampled randomly and checked for sterility by the direct inoculation technique. The vials were stored carefully, until they were to be used, so as to prevent any contamination.

The LDPE stoppers were received in presterilized condition and were therefore used as such. They had been sterilized by the manufacturer by exposing them to gamma-radiation. However, they too were tested for sterility, by the direct inoculation technique, before use. They were stored in a well closed sterile container so as to prevent any contamination.

RESULTS & DISCUSSION:

The **glass vials** were found to comply with the USP specifications for type-1 glass. The heat sterilized vials when tested for sterility were found to be sterile.

The LDPE stoppers were received in presterilized condition. They too were tested for sterility and were found to be sterile.

Direct inoculation technique is the most efficient method of assessing sterility of materials, since indirect methods may fail to retrieve microbes if they are present in small numbers or may give a false positive result if contamination occurs during the test procedure. On the other hand, the direct inoculation technique would reveal the true picture even if the microbial load was relatively low. Additionally, chances of contamination, while performing the test are much lower with this technique

since it only involves direct transfer of test material to the microbiological medium.

2.4.2 PROCEDURE FOR WASHING AND STERILIZATION OF LACQUERED ALUMINIUM TUBES AND POLYPROPYLENE CAPS:

EXPERIMENTAL:

The lacquered aluminium tubes and polypropylene screw caps used for dispensing the ointment and gel were sterilized using the following standard procedure, the entire procedure was carried out in a laminar flow hood:

(i) The ointment tubes and caps were rinsed thrice with 70% v/v isopropyl alcohol.

(ii) The rinsed tubes and caps were completely immersed in a 0.1% w/v solution of benzalkonium chloride in 70% v/v isopropyl alcohol for a period of 4 hours.

(iii) The excess benzalkonium chloride was removed by rinsing several times with sterile 70% v/v isopropyl alcohol. The final rinse was given with sterile isopropyl alcohol.

(iv) The tubes and caps were then kept in a tray in the laminar flow hood for drying overnight.

After drying, the tubes and the caps were tested for sterility using the direct inoculation technique.

RESULTS & DISCUSSION:

The lacquered aluminium tubes along with their polypropylene caps too were checked for sterility and were found to be sterile.

The non-sterile and uncleaned containers can contribute to microbial and particulate contamination to the formulation and hence need to be thoroughly cleaned and sterilized before use. The sterilized containers may not be used immediately, hence they have to be stored in such a way that contamination does not occur. For this reason, these tubes were stored in a sterile stainless steel box with an air tight lid.

2.4.3 PREPARATION OF THE LAMINAR FLOW HOOD BEFORE COMMENCING FORMULATION WORK:

EXPERIMENTAL:

The aseptic room, which houses the laminar flow hood was fumigated with formaldehyde and potassium permanganate, 48 hours prior to starting the formulation work. The UV light attached in the laminar flow was turned on about 3 hours in advance so as to destroy any microbes present on the bench area. The laminar flow was turned on immediately after the UV light had been turned off. Formulation work was commenced atleast 30 minutes after turning on the laminar flow. Just prior to starting work, the laminar flow bench was wiped with sterile 70% isopropyl alcohol, using a fibre-free cloth.

While working on the laminar flow bench, two agar plates containing soyabean-casein digest medium were kept at the left and right corners of the bench. One additional plate was kept on top of the laminar flow bench. The plates were exposed from the

beginning of the formulation work till the end.

RESULTS & DISCUSSION:

Extreme precautions were taken to avoid contamination at all stages of the preparation of dosage forms. The agar plates exposed to the laminar flow bench were found to be sterile on incubation. The plate kept above the laminar flow bench too showed absence of microbial growth.

Monitoring the sterile area for presence of microbes is essential because, if the product that was prepared in that area fails the sterility test, then the formulator should know the source of microbial contamination. If the air in the sterile area was contaminated it would show presence of microbial colonies on the exposed agar plates. If these plates remain sterile, then it would imply that the process, equipment or raw material were contaminated. Thus, validation of all the procedures involved in the preparation of sterile dosage forms was necessary and was therefore undertaken.

2.4.4 PROCEDURES FOR PREPARATION OF INDIVIDUAL DOSAGE FORMS:

The methods of preparations and machines utilized were simple to use and handle. The solutions were prepared in a batch size of 1 litre, the gel was prepared in a 1kg batch, however, in case of the ointment, only a 100gm batch could be prepared.

2.4.4.1 0.3% W/V CIPROFLOXACIN OPHTHALMIC SOLUTION:

EXPERIMENTAL:

Formula:

S.No.	Ingredient	Percentage
1	Ciprofloxacin HCl USP	0.35% w/v
2	Disodium EDTA IP	
3	Benzalkonium chloride solution IP	
4	Sodium acetate trihydrate IP	
5	Glacial acetic acid IP	
6	Sodium chloride IP	
7	Freshly double distilled water to	100%

METHOD:

This formulation being a simple solution, was prepared by dissolving the ingredients one after another in freshly double distilled water and adjusting the pH to 4.5 with glacial acetic acid. The solution was sterilized by membrane filtration (0.22µm). Sterile type-1 amber glass vials of 10mL capacity were used for dispensing the solution. The solution was filled in the vials under laminar flow and the vials were capped with presterilized LDPE plugs. The vials were then sealed with aluminium crimps.

RESULTS & DISCUSSION:

This is a simple solution, and was thus prepared by mixing together all the additives and sterilizing the final solution by membrane filtration. Although CipHCl solution can withstand autoclaving, the final solution could not be autoclaved in the vials as ciprofloxacin tended to crystallize out in the form of fine needles on cooling.

The incorporation of acetate buffer greatly minimized the precipitation of CipHCl at the mouth of the vial, which is a common problem with its marketed counterparts. This buffering would also prevent minor changes in pH from occurring.

Sodium chloride is a widely used tonicity adjusting agent, is inexpensive and easily available, hence was chosen in our formulations.

BKC is the most widely used preservative in ophthalmic preparations because of its potency, broad spectrum of activity and effectiveness over a wide range of pH.

The pH was adjusted to 4.5 because CipHCl solubilizes at acidic pH and on increasing the pH to about 4.8 or slightly more, it begins to crystallize out in the form of fine needles. The official limit for pH of the ophthalmic solution in the USP XXIII is from 3.5-5.5.

2.4.4.2 0.3% W/W CIPROFLOXACIN OPHTHALMIC OINTMENT:

EXPERIMENTAL:

Formula:

S.No.	Ingredient	Percentage
1	Ciprofloxacin HCl USP	0.35% w/w
2	Benzalkonium chloride IP	
3	α-tocopherol NF	
4	White soft paraffin IP	qs to 100%

METHOD:

The entire procedure was carried out in a laminar flow hood. About 250gm of white soft paraffin, to the bulk of which α-tocopherol had been added, was heated to 90°C and was filtered through a sterile 0.22µm membrane filter to be sterilized. 99.65gm of this was separated on a sterile mixing tile. Micronized BKC and the EtO sterilized CipHCl was weighed and transferred on the sterile tile. Both the powders were mixed together using a sterile spatula. The sterile paraffin was mixed with the drug powder in arithmetic proportions by the technique of levigation, till all the paraffin was incorporated.

Before filling the ointment into the tubes, 6 samples of approximately 0.2gm were sampled from different locations and assayed for CipHCl to ensure content uniformity. Only after ensuring content uniformity, filling was undertaken. The ointment was filled into the tubes with the help of a sterile spatula. The tubes were then sealed and crimped.

RESULTS & DISCUSSION:

Ophthalmic ointments, unlike other ointments are sterile preparations and should be prepared with sterile ingredients. CipHCl was successfully sterilized by exposing it to EtO gas.

Various ophthalmic ointment bases are available, and a majority of them utilize white soft paraffin as the principal ingredient. Other base modifiers such as liquid paraffin, beeswax, cetostearyl alcohol, woolfat etc. are also added to serve specific purposes eg. liquid paraffin is added to aid in softening of the petrolatum when applied to the eye, however, the liquid paraffin often separates out and was therefore not used. Woolfat and beeswax are reported to be incompatible with BKC and were hence not used. Cetostearyl alcohol is usually added as a stiffening agent. In our case we chose to use white soft paraffin alone, stabilized with α-tocopherol.

White soft paraffin or petrolatum can be sterilized by either dry heat or gamma-radiation or by membrane filtration. In some instances, dry heat sterilization, at 190°C, has been reported cause oxidation and discolouration, on the other hand gamma-irradiation has also been reported to cause discolouration and 'swelling' of petrolatum. Thus, membrane filtration method was used. Since the petrolatum was intended to be used for ophthalmic products, it had to be free from particulate matter and would have to undergo filtration in any case. Thus, it also supported sterilization by membrane filtration. The only problem with this method is that the petrolatum needs to be heated to liquefy it so as to render it filterable and the need of a steam jacketed

funnel to keep it in the molten state.

2.4.4.3 0.3% W/W CIPROFLOXACIN OPHTHALMIC GEL (LONG-ACTING):

EXPERIMENTAL:

Formula:

S.No.	Ingredient	Percentage
1	Ciprofloxacin HCl USP	0.35% w/w
2	Benzalkonium chloride solution IP	
3	Disodium EDTA IP	
4	Poloxamer 407 NF	
5	Sodium chloride IP	
6	Freshly double distilled water	qs to 100%

METHOD:

The entire procedure was carried out in a laminar flow hood. The "cold method" of preparation of gel was followed. Poloxamer was added to cold water (~5°C) under stirring and was allowed to hydrate completely. The other ingredients were dissolved in freshly double distilled water and added to the polymer solution. The gel was sterilized by autoclaving. It was filled into 10mL amber coloured vials and 5gm capacity lacquered aluminium tubes with the help of a sterile syringe by liquefying it at about 15°C. The vials were capped with presterilized LDPE stoppers and crimped. The tubes were sealed with presterilized polypropylene caps.

RESULTS & DISCUSSION:

Various polymers were evaluated for compatibility with CipHCl. Non-ionic polymers such as HPMC and Poloxamer proved to be compatible with CipHCl. High viscosity grades of HPMC and other cellulose based polymers do form clear transparent gels, however, they also cause matting of eye-lids on drying, which is a major drawback to their use at higher concentrations. Moreover, their solubility decreases with temperature and they precipitate out on autoclaving and would have to be redispersed on cooling. During this cooling phase, the precipitated polymer fibres may initiate crystallization of one of the additives or the drug itself.

Carbopol is a synthetic acrylic acid polymer that is also widely used in ophthalmic preparations. However, it could not be used because of its incompatibility with CipHCl.

Poloxamers, are non-ionic co-polymers, made up of polyoxyethylene-polyoxypropylene monomer blocks. They form clear transparent gels, which are autoclavable and thermoreversible in nature. The thermoreversible property of these gels can be attributed to the formation of hydrogen bonds between water molecules and the ether oxygen atoms of the polymer, at room temperatures and breaking of this bond at lower temperatures[245]. Ophthalmic preparations containing Poloxamers as viscosity building agents are available in the USA. Gels prepared using Poloxamers exhibit pseudoplastic behaviour or shear thinning which is highly desirable, since it matches the rheological properties of natural tears[246,247]. Moreover, Poloxamer-407 was found to be compatible with CipHCl, it was therefore chosen to be

used in the formulation.

In order to determine the optimum polymer concentration to be used in the formulation, it was added at different concentrations to Ciprofloxacin conventional solution and its liquefaction-gelling behaviour was studied. The results are listed in Table-18.

TABLE-18: RESULTS OF THE LIQUEFACTION AND GELLING BEHAVIOUR OF GELS PREPARED WITH VARIOUS CONCENTRATIONS OF POLOXAMER:

CONCENTRATION OF POLOXAMER	NATURE OF SOLUTION	LIQUEFICATION ON COOLING	GELLING AT 37°C
15% w/w	Viscous solution	----	---
18% w/w	Gel	Rapid	Incomplete, fails to gel at times
20% w/w	Gel	Rapid	Gels completely
22% w/w	Gel	Slower	Gels completely
25% w/w	Gel	Very slow, Fails to liquefy at times	Gels completely

Based on the above results, it was decided to use 20% w/w Poloxamer-407, in the formulation.

Since it is a semisolid formulation, which liquefies on cooling, it was dispensed in lacquered aluminium tubes as well as in glass vials, so that it could be applied in the form of an extruded ribbon or could be instilled as a cold drop, which would gel on contact with the eye.

Poloxamers are reported to be incompatible with polyhydroxy compounds such as mannitol, glycerol, sorbitol, polyethylene

glycols etc., hence sodium chloride was used as the tonicity adjusting agent.

Poloxamer gels can be prepared by the cold as well as the hot process, i.e. they can be dissolved in cold or hot water. The cold process is however the preferred method and is also recommended by the manufacturers, since there is a problem of air entrapment and severe foaming with the hot process. Therefore, the cold process was utilized for preparing the long-acting gel.

2.4.4.4 0.3% W/V CIPROFLOXACIN AND 0.1% W/V DEXAMETHASONE OPHTHALMIC SOLUTION:

EXPERIMENTAL:

Formula:

S.No.	Ingredient	Percentage
1	Ciprofloxacin HCl USP	0.35% w/v
2	Dexamethasone IP	0.1% w/v
3	Benzalkonium chloride solution IP	
4	Disodium EDTA IP	
5	Hydroxypropyl-β-cyclodextrin	
6	HPMC E4M (2910 NF)	
7	Sodium acetate.$3H_2O$ IP	
8	Glacial acetic acid IP	
9	Sodium chloride IP	
10	Freshly double distilled water	qs to 100%

METHOD:

CipHCl, BKC, EDTA and sodium chloride were dissolved in a quantity of the acetate buffer solution. Separately, a quantity of the buffer was heated to 90°C and hydroxypropyl-β-cyclodextrin was dissolved in it, subsequently, dexamethasone was added to it in parts, under stirring. Stirring was continued till all of the drug had dissolved, after which HPMC was added and allowed to disperse. Once a uniform dispersion was obtained, it was immediately cooled to ~5°C with stirring. This solution was added to the CipHCl solution, volume was made up and the resulting solution was sterilized by membrane filtration (0.22μm). This sterile solution was filled into sterile type-1 glass amber coloured vials. The vials were capped with presterilized LDPE stoppers and the sealed with aluminium crimps.

RESULTS & DISCUSSION:

Dexamethasone is practically insoluble in water. Although surfactants have been reported to solubilize dexamethasone, by the process of micellar solubilization, very high concentrations of surfactants are required. We found that 6%w/v of tween 80 or Cremophor-EL were required to solubilize 0.1% w/v dexamethasone completely. Using such high concentrations of surfactants in ophthalmic preparations is permitted as these can be tolerated by the eye upto a concentration of 10% w/v. However, there are other problems associated with their use, such as, neutralization of the antimicrobial property of the preservative as well as antibiotic and also generation of excessive foam during their

preparation.

Cyclodextrins are cyclic hexamers or heptamers of monosaccharides, which have a hydrophobic cavity and a hydrophilic exterior, which makes them highly water soluble. These form inclusion-complexes with hydrophobic drugs by incorporating the drug in its hydrophobic cavity and holding it there with the help of weak hydrogen bonds and van der Waals interaction. The drug-cyclodextrin complex is water soluble.

Various cyclodextrins are available, such as α-, β- and gamma-cyclodextrins, depending upon the number of monosaccharides present in the ring. These were further derivatized to form alkyl derivatives such as dimethyl-β-cyclodextrin, hydroxypropyl-β-cyclodextrin, etc. Amongst these hydroxypropyl-β-cyclodextrin is the safest and there are reports on its lack of ocular toxicity[248,249]. It has been reported that the eye can easily tolerate upto 12.5% w/v solution of hydroxypropyl-β-cyclodextrin and that it can be used as an additive in eye-drops. In our formulation, we have used much less than 12.5%. Furthermore, hydroxypropyl-β-cyclodextrin neutralizes the preservative efficacy of highly lipophilic preservatives, such as parabens, chlorbutanol etc. but does not affect that of benzalkonium chloride[250]. Although there are reported methods for the solubilization of dexamethasone by hydroxypropyl-β-cyclodextrin, we optimized the concentration in our laboratory. HPMC E4M was used in this formulation with dual purpose. Firstly, HPMC stabilizes the dexamethasone-hydroxypropyl-β-cyclodextrin complex[243] and secondly it acts as a viscosity imparting agent,

so as to retain the drop in the eye for a slightly longer duration, giving time for the complex to break so as to liberate dexamethasone in the eye.

2.4.4.5 0.3% W/V CIPROFLOXACIN AND 0.1% W/V DEXAMETHASONE OPHTHALMIC SUSPENSION:

EXPERIMENTAL:

Formula:

S.No.	Ingredient	Percentage
1	Ciprofloxacin HCl USP	0.35% w/v
2	Dexamethasone IP	0.10% w/v
3	Benzalkonium chloride solution IP	
4	Disodium EDTA IP	
5	HPMC E4M (2910 NF)	
6	Sodium acetate.$3H_2O$ IP	
7	Glacial acetic acid IP	
8	Sodium chloride IP	
9	Freshly double distilled water	qs to 100%

METHOD:

The entire procedure was carried out in a laminar flow hood. To a sterile and concentrated solution of BKC and HPMC, sterile dexamethasone powder (sterilized by heating at 140°C for 3 hours *in vacuo*) was added under stirring. CipHCl, EDTA and sodium chloride were dissolved in rest of the buffer solution, and the solution was sterilized by autoclaving at 121°C for 15min. at

15psi. The sterile ciprofloxacin solution was added to the sterile suspension and stirring was continued. After making up the volume, the solution was filled in sterile type-1 glass amber coloured vials, capped with LDPE stoppers and sealed with aluminium crimps.

RESULTS & DISCUSSION:

Dexamethasone, unlike CipHCl, can withstand heating to a certain extent. Thus dexamethasone powder was sterilized in a vacuum oven at 140°C for 3 hours.

Most marketed ophthalmic suspensions containing dexamethasone incorporate tween 80 to wet the powder. However, the formula developed by us contains two ingredients which have surface-active properties, namely, BKC as well as HPMC. Thus, the sterile dexamethasone powder was suspended in a sterile solution of these 'surfactants'. The solution of other additives along with CipHCl was sterilized separately and mixed aseptically with this sterile suspension.

Micronized dexamethasone was used to prepare the suspension. If the particle size of the suspended agent exceeds 50µm, it can cause severe eye irritation. The mean particle size (±s.d.) of the dexamethasone powder was 5.20±2.82µm.

In case of the suspension too, sodium chloride was used to adjust the tonicity. This was because it would serve the dual purpose of tonicity adjuster as well as a flocculating agent. Being an electrolyte it could serve the latter purpose also. Various phosphate salts could have been used, but they require pH

adjustment and would result in formation of a strong buffer, which would be irritating to the eye. Sodium chloride does not affect the pH and hence was the salt of choice.

HPMC E4M, F4M and K4M grades are recommended by the manufacturer to be used for ophthalmic preparations. For our formulation we chose HPMC E4M because it provides maximum optical clarity to the solution and is the grade of choice when filtration is to be performed, since it filters with great ease.

2.5 EVALUATION OF THE PREPARED DOSAGE FORMS:

All dosage forms that were investigated had to undergo thorough evaluation. The evaluation included assessment of physicochemical parameters such as drug content, stability, clarity, resuspendability, pH and *in-vitro* release profile; microbiological evaluation included assessment of sterility and efficacy of the antimicrobial preservative; the biological evaluation included assessment of the eye-irritation potential. The aqueous humour drug concentration-time profile was determined for the formulations containing CipHCl only and a suitable animal model was developed to evaluate the efficacy of the eye-drops containing combination of ciprofloxacin and dexamethasone.

2.5.1. PHYSICOCHEMICAL CHARACTERIZATION:

The various physicochemical parameters that were determined or evaluated were: drug content, preservative content, clarity, discolouration, resuspendability, sedimentation rate, particle size distribution of dexamethasone and CipHCl powders, pH the

preparations, viscosity of the gel, *in-vitro* release profile of CipHCl from the gel and accelerated stability studies.

2.5.1.1 Determiantion of initial drug content in all preparations:

EXPERIMENTAL:

The drug content of the formulations were determined (n=6) immediately after their preparation. The methods for determining the drug content were the same as the stability-indicating methods. The assays were carried out as follows:

(i) **Ciprofloxacin ophthalmic solution:** 100µL of the formulation was pipetted out in test tubes and evaporated to dryness *in vacuo* at 45°C. The residue was reconstituted in 5mL of 0.1M orthophosphoric acid, and 20µL was injected into the HPLC.

(ii) **Ciprofloxacin ophthalmic gel:** 200mg of the gel was directly transferred to a 25mL volumetric flask and was dissolved in 5mL of double distilled water. The volume was made up to the mark with more double distilled water, and 20µL of this was injected into the HPLC.

(iii) **Ciprofloxacin ophthalmic ointment:** 200mg of the ointment was directly weighed in a tared 25mL stoppered conical flask. 5 mL of chloroform was added to dissolve the ointment. 10mL of 0.1M orthophosphoric acid was added and the flask was vortexed for 2 minutes and then it was shaken vigorously, periodically for 10 minutes. 20µL of the acidic layer was injected directly into the HPLC.

(iv) **Ciprofloxacin and dexamethasone ophthalmic solution and suspension:** 200µL of the solution and that of the well shaken suspension was pipetted out in test tubes and evaporated to dryness *in vacuo* at 45°C. The residue was reconstituted in 4mL of a 50:50 v/v mixture of methanol and distilled water and 20µL of it was injected into the HPLC.

RESULTS & DISCUSSION:

The initial drug content of all the prepared formulations was measured by the HPLC method and the results are given in Table-19:

TABLE-19: RESULTS OF THE INITIAL DRUG CONTENT OF THE VARIOUS PREPARATIONS:

Formulation		Mean drug content (n=6)
Cip. solution		99.34±0.67
Ciplox^{R}		100.27±0.64
Cip. gel		100.26±0.74
Cip. ointment		104.78±1.57
Cip. + Dexa. Suspension	CIP	97.09±1.31
	DEXA	93.26±0.95
Cip. + Dexa. Solution	CIP	98.74±0.92
	DEXA	95.51±1.41

Ciprofloxacin ophthalmic solution is official in the USP and the limits for content of ciprofloxacin are: not less than 90.0 and not more than 110.0%. The ciprofloxacin content of the prepared solution was within these limits. All other preparations are non-pharmacopoeial.

2.5.1.2 Initial preservative content of all preparations:

EXPERIMENTAL:

Benzalkonium chloride, the preservative used in all the preparations was assayed by the standardized HPLC method. The concentration of the preservative, was also determined after the formulations were prepared. In case of the aqueous preparations, 200µL of the formulation was pipetted out using a calibrated micropipette and was diluted with 600µL of double distilled water, before injecting it in the HPLC. In case of the ointment, 200 mg of the ointment was dissolved in 2mL of chloroform and this was extracted with 800µL of 0.1M orthophosphoric acid. For the purpose of extraction, the solutions were vortexed for a period of 2 minutes and then shaken with the hand periodically over a 10 minute period. 20µL of this solution was injected directly onto the HPLC column.

RESULTS & DISCUSSION:

The results of the assay of benzalkonium chloride are listed in Table-20.

The USP recommends that the preservative should be assayed from the formulations and its efficacy should also be assessed by challenging with test microbes. However, no limits have been specified for the limit of their content. Benzalkonium chloride was reported to produce ocular toxicity at higher concentrations and hence its use was restricted to a maximum of 0.01% w/v by the Indian FDA.

TABLE-20: RESULTS OF THE INITIAL ASSAY (AS % OF THE LABEL CLAIM) OF BKC FROM VARIOUS PREPARATIONS:

Formulation	Mean % preservative content (n=6)
Cip. solution	95.30±7.75
Ciplox^R	31.30±6.17
Cip. gel	92.49±3.30
Cip. ointment	92.10±6.32
Cip. + Dexa. Suspension	93.84±4.90
Cip. + Dexa. Solution	91.26±3.17

2.5.1.3 Evaluation of clarity of the prepared solutions:

EXPERIMENTAL:

Clarity of the solution was checked by visual inspection of the filled vials against black and white backgrounds, under fluorescent white light. In order to detect fine particles or fibres, a drop of the solution was placed on a coverslip which was then inverted on a cavity slide, for observation under a microscope at a magnification of 450X.

RESULTS & DISCUSSION:

All of the clear solutions, namely, Cip. Solution, Cip. + Dexa. solution as well as Cip. gel were found to be free from particulate matter on visual observation as well as when observed under the microscope. The suspension was allowed to settle and the supernatent was visualized for presence of particulate matter. It was then agitated and observed for the presence of coloured particles, no such particles were found.

Clarity of solution is one of the prime requirements of ophthalmic solutions. Lack of clarity can be due to haziness in solution, microbial growth or presence of particulate matter. Further, haziness in solution can also be due to reaction of the contents and the container, on the other hand particulate matter can be introduced from the environment, equipment used in preparation and the raw material itself. Lack of clarity can thus point to many detrimental interactions and was hence evaluated.

2.5.1.4 Evaluation of resuspendability of the Cip + Dexa suspension:

EXPERIMENTAL:

The ability of the settled suspension to be resuspended on mild agitation was assessed by inverting the vial containing a settled suspension 10 times. If this was insufficient to resuspend the suspension, the number of inversions required to resuspend it were noted. After resuspension was effected, the suspension was observed under a low power microscope for the presence of particle aggregates. Resuspendability was also checked at 1, 2, 3, 5 and 6 months intervals during accelerated stability studies.

RESULTS & DISCUSSION:

The ciprofloxacin and dexamethasone suspension was found to be easily resuspendable after standing undisturbed for as long as 6 months at 5°C and 25°C only. The settled particles could be easily dispersed by simply inverting the vial 6-8 times. At 37°C and 45°C, the settled dexamethasone particles clumped together to

form a non-dispersible cake.

Redispersibility or resuspendability is an important parameter to be evaluated in case of suspensions. The suspended particles tend to settle down upon standing, due to gravity and while administering the dose, it needs to be shaken so as to redisperse it uniformly to ensure that the same dose is dispensed every time.

2.5.1.5 Determination of the sedimentation rate of dexamethasone particles in Cip + Dexa suspension.

EXPERIMENTAL:

A well shaken suspension was poured in a 10mL measuring cylinder which was then placed on a flat surface, undisturbed so as to study the settling of dexamethasone particles. If the settling rate could not be measured, the time for the particles to settle completely was noted.

RESULTS & DISCUSSION:

The process of settling could not be studied well in case of this suspension because the particle size of dexamethasone was so small, that a clear supernatent could not be observed. Moreover, the concentration of dexamethasone in the suspension was only 0.1% w/v, which too made it difficult to measure sedimentation rate. However the time for complete settling of the particles and to obtain a clear supernatent was about 48 hours.

2.5.1.6 Determination of the particle size distribution of CipHCl powder to be used in preparation of the ointment and dexamethasone powder to be used in the suspension:

EXPERIMENTAL:

The particle size distribution of the dexamethasone suspension was found by using an eye-piece micrometer along with a light microscope. For this purpose, a drop of a well shaken suspension was placed on a slide and was covered with a coverslip and the lengths of 300 different particles was measured. Micronized CipHCl powder, intended to be incorporated into the ointment was also evaluated for its particle size distribution, for which it was suspended in light mineral oil and a drop of this suspension was placed on a slide. The lengths of 300 particles were measured.

RESULTS & DISCUSSION:

The results of the measurements of particle size of CipHCl and dexamethasone powders are shown in Table-21:

TABLE-21: RESULTS OF THE PARTICLE-SIZE DISTRIBUTION OF CIPHCl POWDER, DEXAMETHASONE POWDER AS WELL AS DEXAMETHASONE IN SUSPENSION:

	Mean ± s.d. (n=300)
Ciprofloxacin HCl powder	4.68±2.14µm
Dexamethasone powder	5.20±2.82µm
Cip + Dexa suspension Initial:	5.23±2.72µm

The results indicate that the particle size of dexamethasone remained unchanged, immediately after incorporation into suspension, which reflects that the vehicle did not cause aggregation of particles.

2.5.1.7 Determination of pH of the preparations:

EXPERIMENTAL:

The pH of the contents of two randomly sampled vials was measured using a standardized pH meter immediately after preparation of the formulation, as well as at the end of 1, 3 and 6 months of stability studies.

RESULTS & DISCUSSION:

The results of the measurements of pH of the various ophthalmic solutions just after their manufacture, were as follows:

TABLE-22: RESULTS OF pH MEASUREMENTS OF THE PREPARATION, ON THE DAY OF THEIR MANUFACTURE:

Formulation	pH
Cip. solution	4.48
Cip. gel	4.52
Cip. + dexa suspension	4.48
Cip. + dexa solution	4.46

The pH values were found to be close to their expected value of 4.50.

2.5.1.8 Determination of viscosity of the gel:

EXPERIMENTAL:

The viscosity of the Poloxamer gel was determined using a Brookefield viscometer, using spindle T-F and the helipath stand, at 25°C and various spindle speeds.

RESULTS & DISCUSSION:

The results of viscosity measurements are summarized in Table-23:

TABLE-23: VISCOSITY MEASUREMENTS OF CIP. GEL AT 25°C, AT DIFFERENT SPINDLE SPEEDS:

Viscometer Spindle speed (rpm)	Measured viscosity (centipoises)
0.3	484250
1.0	328500
2.5	183150
5.0	103650
10.0	58065
20.0	31240
50.0	13430
100.0	7650

These results suggest that the gel exhibits pseudoplastic behaviour or shear thinning since the viscosity decreases with increasing shear. Bothner *et al*[247] suggested that a tear substitute should have shear thinning properties as do natural tears. The low viscosity at high shear rates produces lubrication during blinking and the high viscosity at zero shear rate prevents the fluid from flowing away from the cornea when the eyelids are not blinking.

2.5.1.9 Determination of *in-vitro* drug release profile from the long acting gel:

EXPERIMENTAL:

The *in-vitro* drug release was studied using a USP type 5 dissolution test apparatus, as was reported by Rozier *et al*[98]. As per the procedure, 1 gm of the gel or the ointment was placed a 50mm glass pertiplate. The gel was spread evenly in the petri plate whereas the ointment had to be kept in the form of cylindrical segments (the form in which it was extruded out of the tube). These petriplates were then covered with a 30# nylon mesh. Phosphate buffered saline IP (400mL), maintained at 37°C was used as the dissolution medium. The paddle speed was 30 rpm. 5mL aliquots were withdrawn at intervals of 2.5, 5, 10, 15, 20, 30, 45 and 60 minutes. After each aliquot was withdrawn, it was replaced with an equal volume of fresh dissolution medium. The concentrations of ciprofloxacin in the aliquots were then estimated by measuring their absorbance at 274nm. Each experiment was performed in triplicate. *In-vitro* release was also studied from gels prepared with different polymer concentrations, viz., 15, 18, 20, 22 and 25% w/w.

Composition of Phosphate buffered saline, pH 7.4 IP:

S.No.	Ingredient	Quantity
1	Disodium hydrogen orthophosphate.12H_2O	2.38 gm
2	Potassium dihydrogen orthophosphate	0.19 gm
3	Sodium chloride	8.00 gm
4	Water	qs to 1 lit

RESULTS & DISCUSSION:

No drug was released from the ointment even after 5 hours of immersion in the dissolution medium. This was probably due to the fact that the ointment needs to be sheared so as to enable it to release the drug, otherwise the drug will be covered by the hydrophobic ointment base and would not release the drug at all. In the eyes, the shearing force is provided by the blinking action. However, there was no shearing force in the dissolution medium and hence no drug could be released.

The drug release was measured from the gels, which had been prepared with different polymer concentrations. The plot of cumulative percent released versus time is shown in Fig. 10. The conventional solution as well as the viscous solution containing 15% w/w of Poloxamer, released the drug rapidly. In case of the conventional solution, drug was released in 2.5 minutes itself. More than 95 % of the drug was released form the viscous solution in 10 minutes. The drug release from the gels with polymer concentrations of 18, 20, 22, and 25 % w/w followed Higuchi kinetics i.e. the plot of cumulative percent released versus square root of time was linear. This indicated that the drug release was occurring purely by diffusion. The release data when fitted to the Power Law Expression,

$M_t/M_\infty = Kt^n$ Where M_t = Amount of drug released at time t
M_∞ = Amount of drug released at time ∞
K = Constant
n = Diffusion coefficient
t = time

gave values of 'n' close to 0.5, which too suggested that the release was diffusion controlled. The values of 'n' and the t_{90} (time taken to release 90% of the drug present in the gel) values at different polymer concentrations are summarized in Table-24.

TABLE-24: T_{90} VALUES OBTAINED FROM THE RELEASE PROFILE DRUG FROM OF THE GEL AT DIFFERENT POLYMER CONCENTRATIONS:

Polymer concentration	'n'	t_{90}
15% w/w	0.3494	09.22 min
18% w/w	0.5790	23.41 min
20% w/w	0.5487	30.32 min
22% w/w	0.5550	32.60 min
25% w/w	0.6018	35.48 min

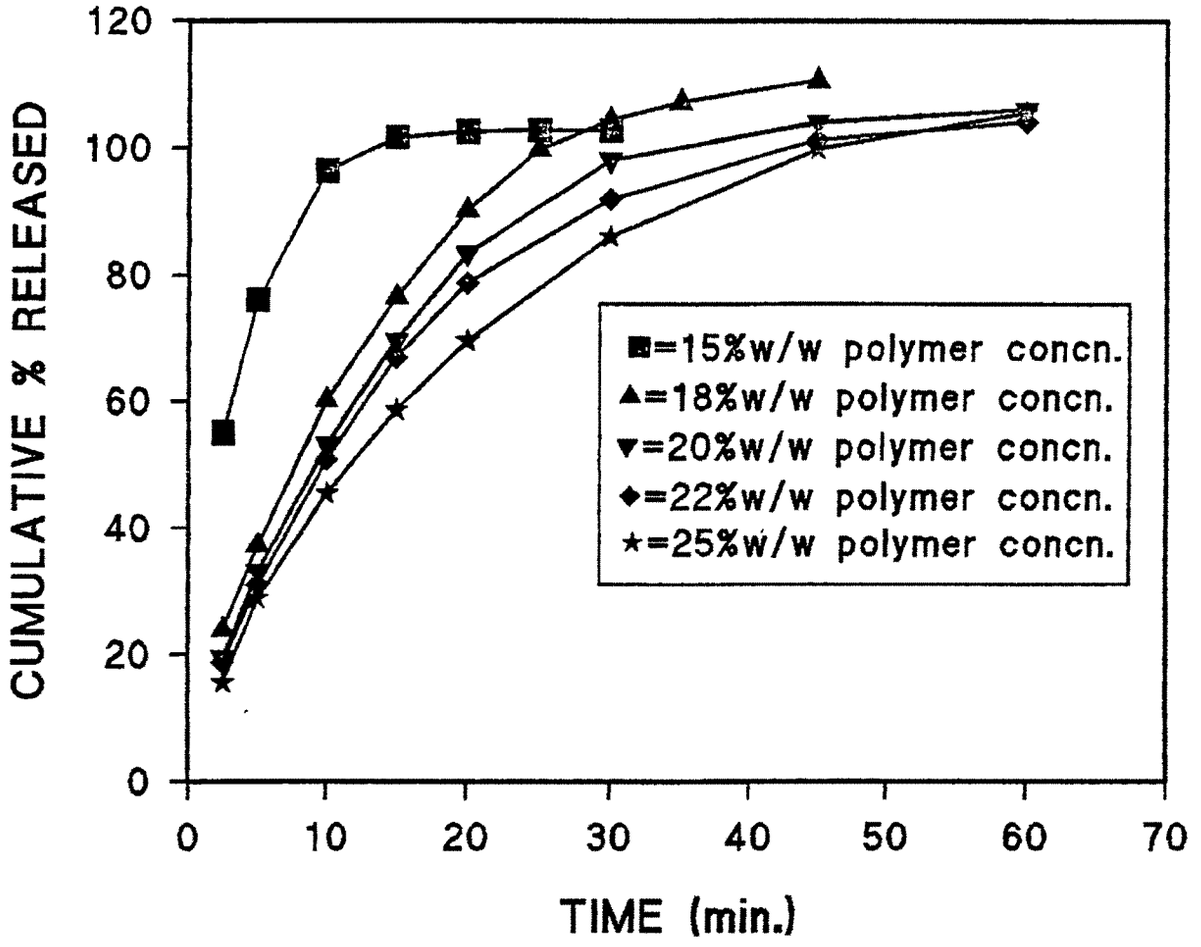

Fig. 10

In vitro release of ciprofloxacin from poloxamer gels: Effect of increasing polymer concentration on release profile

2.5.1.10 Accelerated stability studies:

EXPERIMENTAL:

All the formulations in their final form were subjected to accelerated stability studies, at 5°C, 25°C, 37°C, 45°C, 37°C/75% RH and also accelerated conditions of light. In order to accelerate the conditions of exposure to light, the vials were placed on the window sill where direct sunlight would be incident on them. Accelerated stability studies were carried out for a period of 6 months. The drug contents were measured by their respective stability-indicating methods at intervals of 1, 2, 3, 5, and 6 months. For the purpose of assaying drugs, a 3-point standard curve was developed on each day of analysis, prior to injecting the stability samples. Assay of the preservative was carried out at the beginning and end of the accelerated stability studies. The preservative concentration was determined by comparing its areas to a standard solution. Additionally, viscosity of the gel and its *in-vitro* release rates were also determined at the end of the stability period. The tests such as pH of solution, particle size distribution etc. were repeated during the accelerated stability studies. The test procedures for these were the same as those described in Section 2.4. The procedures of only those additional tests that were carried out specifically for the purpose of accelerated stability studies are described.

RESULTS & DISCUSSION:

Tables 25-33 show the data of drug contents of the respective formulations at different time intervals during the accelerated study:

TABLE-25: ACCELERATED STABILITY DATA:
CONTENT OF CIPROFLOXACIN IN CIP. SOLUTION: (n=2)

Condition	Sampling time				
	1month	2months	3months	5months	6months
5°C	99.75 ±0.07	99.50 ±1.13	100.90 ±0.88	98.35 ±0.83	97.90 ±0.70
25°C	99.00 ±1.16	101.23 ±0.83	100.99 ±0.25	98.36 ±0.95	97.18 ±0.89
37°C	99.18 ±0.32	100.69 ±2.16	100.59 ±0.40	101.01 ±0.59	97.70 ±0.11
45°C	99.80 ±0.64	99.36 ±0.95	99.78 -	97.92 ±0.53	98.87 ±0.52
light	101.08 ±1.04	--	--	--	100.47 ±1.85

The content of ciprofloxacin was found to remain constant throughout the period of stability studies. The percentage of Analog-A too did not increase in this duration, at any temperature. There is however no mention of the limit of Analog-A in Ciprofloxacin ophthalmic solution USP. Since there was no significant degradation or loss in content of ciprofloxacin, a shelf-life of 18 months can be assigned to this product.

TABLE-26: ACCELERATED STABILITY DATA:
CONTENT OF CIPROFLOXACIN IN CIPLOX^{R}: (n=2)

Condition	Sampling time				
	1month	2months	3months	5months	6months
5°C	101.06 ±0.48	102.46 ±2.32	106.23 ±0.57	104.98 ±1.28	107.43 ±0.46
25°C	100.31 ±0.60	104.47 ±1.03	105.32 ±2.40	104.17 ±0.61	112.13 ±4.79
37°C	100.68 ±0.09	106.24 ±0.06	109.02 ±1.73	109.63 ±3.51	117.97 ±4.48
45°C	99.65 ±0.13	105.81 ±1.82	104.78 ±5.33	108.99 ±4.80	113.48 ±3.00
light	103.99 ±0.30	--	--	--	107.54 ±1.94

In case of Ciplox^{R}, which was dispensed in 5mL vials, no increase in the content of Analog-A was observed, indicating that the formulation was chemically stable. There was however a significant loss of water, due to vaporization, probably as the LDPE cap was not snug enough to curtail such a loss.

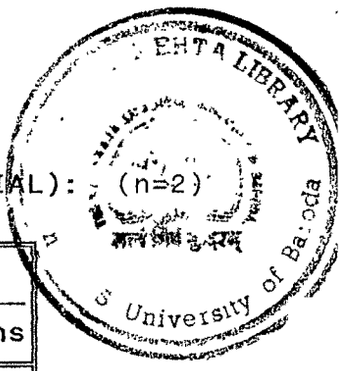

TABLE-27: ACCELERATED STABILITY DATA:
CONTENT OF CIPROFLOXACIN IN CIP. GEL (IN VIAL): (n=2)

Condition	Sampling time				
	1month	2months	3months	5months	6months
5°C	99.72 ±0.11	100.24 ±1.01	98.70 ±0.13	100.46 ±1.91	100.00 ±1.41
25°C	99.27 ±1.39	100.59 ±0.09	99.49 ±0.95	99.63 ±1.44	99.13 ±1.93
37°C	99.41 ±1.78	98.32 ±0.03	98.77 ±0.49	100.18 ±1.51	100.58 ±1.50
45°C	103.17 ±1.91	102.28 ±3.63	99.69 ±0.16	98.70 ±2.38	101.11 ±5.88
light	100.44 ±0.23	--	--	--	98.27 ±0.35

In case of the gel stored in the vial, the content of Analog-A increased to a maximum of 0.40%. The limit of Analog-A, in ciprofloxacin infusion USP is given as ≤ 1.0%. Thus we can claim that no significant degradation had occurred in the gel stored in the vials. A shelf-life of 18 months, which is the maximum for ophthalmic products can be assigned to this formulation.

TABLE-28: ACCELERATED STABILITY DATA:
CONTENT OF CIPROFLOXACIN IN CIP. GEL (IN OINTMENT TUBE): (n=2)

Condition	Sampling time		
	1 month	2 months	3 months
5°C	95.00 ±0.80	86.95 ±0.11	86.54 ±5.58
25°C	85.97 ±0.16	81.08 ±2.71	75.65 ±11.73
37°C	98.51 ±2.86	95.17 ±6.60	88.40 ±10.25
45°C	95.38 ±0.17	94.93 ±3.35	78.94 ±10.84
light	100.08 ±1.05	--	98.27 ±0.38

However, in case of the gel filled in ointment tubes, more than 20% Analog-A was found at the end of three months at 5°, 25° and 37°C, probably due to the interaction between CipHCl and Al metal of the tube. The lacquered Al ointment tube was thus considered to be unsuitable for dispensing Cip. gel.

TABLE-29: ACCELERATED STABILITY DATA:
CONTENT OF CIPROFLOXACIN IN CIP. OINTMENT: (n=3)

Condition	Sampling time				
	1month	2months	3months	5months	6months
5°C	101.16 ±1.72	100.09 ±2.23	100.87 ±1.52	99.52 ±2.26	100.62 ±2.05
25°C	100.98 ±1.05	101.49 ±2.21	100.82 ±3.12	99.78 ±1.89	100.45 ±2.86
37°C	99.93 ±2.11	100.05 ±1.52	99.52 ±2.78	99.37 ±2.41	100.05 ±2.40
45°C	101.41 ±2.03	101.80 ±2.51	101.34 ±2.46	100.81 ±2.10	101.75 ±1.37
37°C/ 75%RH	100.68 ±2.45	99.08 ±2.30	98.62 ±1.94	100.32 ±2.84	98.63 ±1.53

CipHCl is very stable in the solid form and it remained so in the ointment too. There was no loss of the drug at any of the conditions during and at the end of accelerated stability studies. The content of Analog-A, too remained unchanged during this period of 6 months. Since no significant degradation was observed, a shelf life of 18 months can be proposed for this preparation.

TABLE-30: ACCELERATED STABILITY DATA:
CONTENT OF CIPROFLOXACIN IN CIP.+ DEXA. SOLUTION: (n=2)

Condition	Sampling time				
	1month	2months	3months	5months	6months
5°C	101.00 ±0.61	104.35 ±0.52	100.73 ±1.72	101.87 ±4.58	99.52 ±0.39
25°C	102.52 ±0.17	103.73 ±1.99	100.32 ±0.15	103.55 ±1.59	99.75 ±0.13
37°C	100.60 ±3.73	102.69 ±1.02	102.33 ±1.29	103.24 ±0.21	100.24 ±1.87
45°C	101.33 ±0.16	102.67 ±0.25	101.32 ±1.87	105.01 ±1.72	99.85 ±0.16
Light	98.25 ±0.23	--	--	--	103.97 ±0.06

There was no significant change in the assay of ciprofloxacin even after a period of 6 months, at different temperatures. The content of Analog-A, too did not increase during this period. The formulation can be considered stable with respect to ciprofloxacin.

TABLE-31: ACCELERATED STABILITY DATA:
CONTENT OF DEXAMETHASONE IN CIP.+ DEXA. SOLUTION: (n=2)

Condition	Sampling time			
	1month	2months	5months	6months
5°C	100.37 ±0.18	100.59 ±0.49	102.43 ±1.51	100.80 ±0.52
25°C	101.31 ±0.35	99.58 ±1.49	98.72 ±1.31	100.59 ±0.33
37°C	96.94 ±3.65	96.67 ±0.29	95.72 ±1.07	91.40 ±3.77
45°C	95.76 ±0.06	93.45 ±0.95	93.62 ±0.65	85.49 ±6.39
Light	97.88 ±0.14	--	--	97.49 ±1.99

There was no loss of dexamethasone at 5 and 25°C, after a period of 6 months. More than 3-4% of dexamethasone was lost at higher temperatures at the end of 1month, however, no peak of the degradation product was seen in any of these cases, so it was assumed that the drug might have been adsorbed onto the surface of the glass vial.

Since there was a significant loss of the steroid at 37°C and above, it was thought necessary to store the preparation in a refrigerator. The USP demands stability data at the temperature of storage as well as 15°C above this temperature. If we assume the temperature in the refrigerator to be 8-10°C, the 25°C data would support the claim that the preparation is stable under these conditions.

TABLE-32: ACCELERATED STABILITY DATA:
CONTENT OF CIPROFLOXACIN IN CIP.+ DEXA. SUSPENSION:
(n=2)

Condition	Sampling time			
	1month	2months	3months	6months
5°C	101.00 ±1.29	100.23	100.28 ±3.72	102.73 ±3.97
25°C	102.31 ±4.17	101.47 ±2.60	100.50 ±3.25	100.60 ±3.08
Light	97.95 ±1.10	--	--	97.45 ±1.39

In case of the suspension, there was no change in the content of ciprofloxacin. Since no increase in the content of Analog-A was noted, this preparation can be considered stable with respect to ciprofloxacin. There was a slight decrease in the content of ciprofloxacin, on prolonged exposure to light, however this change was not considered to be significant, as per the USP guidelines. Moreover, there was no increase in the content of Analog-A, which means that there was no degradation.

TABLE-33: ACCELERATED STABILITY DATA:
CONTENT OF DEXAMETHASONE IN CIP.+ DEXA. SUSPENSION:
(n=2)

Condition	Sampling time			
	1month	2months	3months	6months
5°C	101.03 ±0.03	100.56 --	99.49 ±1.64	98.41 ±1.62
25°C	102.14 ±4.87	99.91 ±1.12	98.91 ±1.51	98.77 ±1.44
Light	100.82 ±1.05	--	--	100.24 ±1.80

The suspension was found to be physically unstable at 37°C and above. The settled layer of dexamethasone was found to form a non-dispersible cake within a period of 1 month itself. The settled layer or sediment of dexamethasone, however, remained totally and satisfactorily dispersible at lower temperatures. This formulation too should be stored under refrigeration to prevent this instability. The exact cause of such a cake formation could not be ascertained.

TABLE-34: ACCELERATED STABILITY DATA: ASSAY OF BENZALKONIUM CHLORIDE (% OF INITIAL) FROM VARIOUS PREPARATIONS, AT THE END OF 6 MONTHS OF ACCELERATED STABILITY STUDIES: (n=2)

Preparation	5°C	45°C
Cip. solution	94.43±4.31	97.02±1.69
Cip. gel (vial)	93.23±5.35	88.64±8.41
Cip. ointment	87.85±5.27	86.58±7.22
Cip + dexa Suspension	96.10±4.38	93.62±4.89
Cip + Dexa Solution	93.66±1.08	80.82±3.42

The content of BKC was found to be only marginally reduced in the solutions. However, in case of the ointment, a slightly more reduction in assay was observed at both 5°C as well as 45°C. In case of the ointment a moderate reduction would not be considered as significant as chances of contamination are lesser as compared to the solutions. At present there is no regulatory guideline on the limit of content of preservative at the end of accelerated stability studies.

TABLE 35: ACCELERATED STABILITY DATA:
pH OF THE PREPARATIONS AT DIFFERENT TIMES DURING ACCELERATED STABILITY STUDIES:

Preparation	pH at the end of 1month	3months	6months
Cip. solution	4.47	4.47	4.50
Cip. gel (vial)	4.51	4.52	4.52
Cip + dexa Suspension	4.43	4.45	4.45
Cip + Dexa Solution	4.45	4.44	4.46

The pH of all the solutions were found to be fairly constant, throughout the period of stability studies. The addition of buffer might have helped in maintaining the pH close to the actual value of 4.50.

In vitro drug release profile of Cip. gel stored in vial at 5°C, 25°C, 37°C, 45°C and under accelerated conditions of light are given in Table-36. The results indicate that there appeared to be no significant change in the drug release profile after storage at accelerated conditions temperature for a period of 6 months.

TABLE-36: ACCELERATED STABILITY DATA:
DATA OBTAINED FROM THE *IN-VITRO* RELEASE PEOFILE OF CIPROFLOXACIN FROM THE GEL: INITIAL (ZERO DAY) VALUES AND THOSE OBTAINED AFETR STORAGE FOR 6 MONTHS AT DIFFERENT TEMPERATURES. (n=3)

	Mean % drug released±s.e.m.				
Sampling Time in min.	Storage temperature				
	Initial	5°C	25°C	37°C	45°C
2.5	19.55 ±1.08	18.82 ±2.12	20.11 ±2.23	20.89 ±2.47	16.22 ±1.91
5.0	33.02 ±1.54	34.55 ±2.08	34.70 ±2.14	33.93 ±3.05	35.74 ±1.54
10.0	53.10 ±3.67	54.89 ±0.77	52.46 ±2.51	56.73 ±1.14	53.18 ±2.64
15.0	69.70 ±5.99	68.78 ±3.58	72.21 ±4.29	70.41 ±5.40	71.39 ±4.51
20.0	83.27 ±7.62	86.01 ±6.84	86.09 ±7.16	83.36 ±6.73	87.60 ±7.25
30.0	98.04 ±8.00	95.98 ±7.61	97.62 ±8.55	98.78 ±7.28	99.80 ±8.72
45.0	103.99 ±4.80	102.15 ±2.01	104.24 ±2.20	101.38 ±3.58	102.07 ±1.97
60.0	106.02 ±2.47	104.29 ±2.57	105.32 ±2.46	102.79 ±1.54	104.41 ±2.77

The *in-vitro* drug release data suggests that the gel had not changed its properties appreciably and hence the profile remained constant during the stability testing period, at low as well as high temperatures. Some polymers do undergo changes upon aging and this may result in drastic changes in the release profiles. The consistency in release profiles can also be related to the absence of changes in viscosity of the gel on being subjected to

accelerated conditions of temperature (Table-37).

2.5.1.10 Accelerated stability studies: Evaluation of discolouration in the preparations, if any:

EXPERIMENTAL:

Discolouration of the solutions, on being subjected to accelerated conditions of temperature and light, was assessed at the end of 1, 3, 6 months. This was done by pouring out the contents of two vials into a Nessler's cylinder and comparing it visually with a freshly prepared 0.35% w/v solution of CipHCl. In case of the suspension, it was first centrifuged and the supernatent was transferred to a Nessler's cylinder, for comparison. The gel was cooled and the contents were poured out in the Nessler's cylinder.

RESULTS & DISCUSSION:

The formulations were observed periodically for the presence of any discolouration. Since the formulations were filled in amber coloured vials, it was difficult to perceive any difference in colour. Hence, the contents of the vials were emptied into Nessler's cylinders and were observed against a white background. None of the solutions observed showed discolouration. Only the gel filled in the ointment tubes showed a yellow discolouration after storage at 45°C for a period of 3 months. The intensity of discolouration was higher in the gel present at the tip of the tube, however, it was much less in the gel deeper inside.

The probable reason for this discolouration could be the chelation of Al of the ointment tube body by CipHCl and probably

additional oxidation might have taken place at the tip of the tube. On removing the rest of the gel from these tubes, the inner lacquered surface of the tube was examined, which showed that the lacquer had peeled off from the aluminium body at many places and the gel was in direct contact with the metal of the tube.

Discolouration of formulation indicates that some degradative change has occurred. This change can be mostly traced to microbial contamination, oxidative degradation or reaction with the container. Once the source of discolouration is traced, it has to be eradicated, so as to stabilize the formulation.

TABLE-37: ACCELERATED STABILITY DATA:
VISCOSITY OF THE GEL (IN VIAL) AFTER THE COMPLETION OF 6 MONTHS OF ACCELERATED STABILITY STUDIES:

Viscometer Spindle speed (rpm)	Condition of storage				
	Initial	5°C	25°C	37°C	45°C
0.3	484250	502250	442800	452380	468890
1.0	328500	352840	315940	301100	298500
2.5	183150	215640	188520	192120	178950
5.0	103650	122455	115890	116350	99855
10.0	58065	60930	58750	55470	57450
20.0	31240	32100	32550	29980	30850
50.0	13430	14025	12980	12785	12660
100.0	7650	7800	7090	7350	7170

The viscosities at different speeds and after storage at different temperatures for a period of 6 months did not show any significant change, which means that the gels were physically stable and would provide consistent release of drug over a period of time.

TABLE-38: ACCELERATED STABILITY DATA:
PARTICLE SIZE DISTRIBUTION OF DEXAMETHASONE, INITIALLY AND AFTER STORAGE AT 5° AND 25°C FOR A PERIOD OF 6 MONTHS.

Condition	Mean±s.d. (n=300)
Initial	5.23±2.72µm
After storage at 5°C for 6 months	5.76±3.26µm
After storage at 25°C for 6 months	5.37±2.60µm

The results indicate that on storage for 6 months, at 5°C and 25°C, the particle size remained unchanged. Hence it can be said that the suspension was physically stable at 5° and 25°C. However, the suspension tended to cake at 37°C and above. The reason for this instability could not be ascertained. It was therefore recommended to store the suspension in a cool place or preferably in a refrigerator so as to prevent any instability.

Furthermore, the time taken for the Cip + Dexa suspension to settle completely, after storage at 5°C and 25°C for a period of 6 months, was about 48 hours. Which is indicative of the physical stability of the suspension.

2.5.2 MICROBIOLOGICAL EVALUATION:

Amongst the microbiological tests, two tests were carried out, namely the test for sterility and the antimicrobial effectiveness test.

2.5.2.1 Test for sterility:

EXPERIMENTAL:

All ophthalmic solutions are required to be sterile. Although utmost aseptic precautions are taken during their manufacture, it is essential to check the sterility of the final formulations. Sterility of any substance can be assessed by simply inoculating it or a part of it in sterile microbiological nutrient media. If microbes are present, they would multiply in the media and this growth would be indicated by presence of turbidity in the media. When antimicrobial substances are present in the substance under test, the USP specifies the membrane filtration method, in which a solution of the substance is passed through a sterile microbial membrane filter (0.45µm porosity or less), the filter is washed with a sterile solution of minimal media such as peptone and then cut into two pieces. One piece is aseptically transferred to a flask containing sterile fluid thioglycollate broth and the other to a flask containing sterile broth of soyabean casein digest medium, the former supports anaerobic as well as aerobic growth whereas the latter supports the growth of yeast and fungi.

In our case, all the formulations contained 0.3% ciprofloxacin, hence the sterility of all of these was checked by the membrane filtration method. Modifications in the procedure had to be made depending upon the type of preparation (solution, gel, suspension or ointment) as per the official guidelines. The procedures are being briefly described:

(i) Ciprofloxacin ophthalmic solution and ciprofloxacin with dexamethasone ophthalmic solution: The contents of two vials were emptied onto the filter and were filtered with the aid of suction. The filter was washed thrice with a 10 mL portions of 0.1% w/v sterile solution of peptone (Fluid A) in distilled water.

(ii) Ciprofloxacin ophthalmic ointment: In case of the ointment, 1.0gm of the ointment from each of the two tubes were combined and dissolved in 100mL of sterile isopropyl myristate (IPM). The IPM had been rendered sterile by means of membrane filtration. This oily mixture was aseptically transferred to membrane filter funnels and filtered with the aid of suction. Following filtration of the sample, the membrane was washed thrice with 100mL portions of Fluid K and then with 100mL of Fluid A.

Composition of Fluid K:

Ingredient	Quantity
Bacteriological peptone	5gm
Beef extract	3gm
Polysorbate 80	10gm
Water	1000mL

(iii) Ciprofloxacin ophthalmic gel: About 1gm of the gel was transferred directly into 50mL of sterile saline and then aseptically filtered through a sterile membrane filter. The filter was then washed thrice with 10mL portions of

Fluid A.

(iv) Ciprofloxacin with dexamethasone ophthalmic suspension: The contents of two vials were filtered through a sterile membrane filter and the filter was washed thrice with 10 mL portions of Fluid A.

All the membranes were aseptically cut into two halves. One half of it was aseptically transferred to 100mL of sterile fluid thioglycollate medium and the other half to 100mL of sterile soyabean casein digest medium. During the experiment negative and positive controls were also used. For the negative controls, a mock inoculation was performed and in the case of positive controls, live microbes such as *S. aureus* and *A. niger* were inoculated into the medium. All the flasks containing soyabean casein digest medium were incubated at 25°C and fluid thioglycollate medium at 32°C for 7 days. In case of the dexamethasone suspension, a loopful of the culture medium was transferred to fresh medium after 7 days and this freshly inoculated culture was incubated for a further 7 days. At the end of 7 days, the flasks were observed for presence of turbidity.

RESULTS & DISCUSSION:

All the preparations were evaluated for sterility by the USP membrane filtration method and were found be sterile. Sterility is also one of the prime requirements of topical ophthalmic products. Antibiotic eye drops are usually administered when the eye is infected or inflamed, this is a situation when the eye is immunologically compromised. Administration of non-sterile medication in such an eye can only worsen the clinical situation

and may also lead to loss of vision. Thus topical ophthalmic products are not only supposed to be sterile at the time of being dispensed, but as far as possible sterile ingredients should be used in their manufacture so as to reduce the bioburden in them. This will eliminate the toxic microbial products too, which might otherwise cause unwanted toxicity.

2.5.2.2. Antimicrobial preservatives- Effectiveness test:

Antimicrobial preservatives are substances added to dosage forms, especially in multiple dose containers so as as inhibit the growth of microorganisms that may be introduced inadvertently during use. This test demonstrates, in multiple dose parenteral, otic, nasal and ophthalmic products, the effectiveness of any added antimicrobial preservative to control the growth of microbes that may be introduced in the formulation. The tests and standards apply only to the product in original unopened container in which it was dispensed.

EXPERIMENTAL:

The test involves challenging the formulation with atleast 5 test microorganisms, namely, *Aspergillus niger (ATCC 16404), Candida albicans (ATCC 10231), Escherichia coli (ATCC 8739), Pseudomonas aeruginosa (ATCC 9027) and Staphylococcus aureus (ATCC 6538P).* The microorganisms were grown on suitable medium and the cells or spores were harvested with sterile saline and adjusted to a concentration of ~10^8/mL. This suspension was inoculated into the product container such that the formulation contained between

10^5-10^6microorganisms/mL. The formulations were incubated at 25°C and periodically observed at 7, 14, 21 and 28 days for any changes in appearance. Additionally, the number of viable microorganisms remaining at the end of each period was determined by the plate count method. The percentage change in the concentration of each microorganism during the test was determined.

RESULTS & DISCUSSION:

The antimicrobial effectiveness test or preservative efficacy test was carried out as per the guidelines given in the USP. The results are summarized in Table-39:

TABLE-39: RESULTS OF THE PRESERVATIVE EFFECTIVENESS TEST:

Formulation ?	Microbial count as c.f.u. per mL C. A.	A. N.	S. A.	E.C.	P.A.
Cip. Solution					
cfu/mL initial ?	$4.6X10^5$	$1.55X10^5$	$2.05X10^5$	$2.03X10^5$	$2.5X10^5$
Microbial count as % of initial					
Day 7	0.00	0.00	0.00	0.00	0.00
Day 14	0.00	0.00	0.00	0.00	0.00
Day 21	0.00	0.00	0.00	0.00	0.00
Day 28	0.00	0.00	0.00	0.00	0.00
Cip. gel					
cfu/mL initial ?	$4.6X10^5$	$1.55X10^5$	$2.05X10^5$	$2.03X10^5$	$2.5X10^5$
Microbial count as % of initial					
Day 7	0.00	0.00	0.00	0.00	0.00
Day 14	0.00	0.00	0.00	0.00	0.00
Day 21	0.00	0.00	0.00	0.00	0.00
Day 28	0.00	0.00	0.00	0.00	0.00

Contd...

Table 39: contd....

Cip. ointment					
cfu/gm initial	$1.0X10^5$	$3.40X10^5$	$4.56X10^5$	$4.50X10^5$	$5.56X10^5$
Microbial count as % of initial					
Day 7	0.22	0.041	0.00	0.00	0.00
Day 14	0.00	0.002	0.00	0.00	0.00
Day 21	0.00	0.002	0.00	0.00	0.00
Day 28	0.00	0.001	0.00	0.00	0.00
Cip. + Dexa. Solution					
cfu/mL initial	$4.6X10^5$	$1.55X10^5$	$2.05X10^5$	$2.03X10^5$	$2.5X10^5$
Microbial count as % of initial					
Day 7	0.00	0.00	0.00	0.00	0.00
Day 14	0.00	0.00	0.00	0.00	0.00
Day 21	0.00	0.00	0.00	0.00	0.00
Day 28	0.00	0.00	0.00	0.00	0.00
Cip. + Dexa. Suspension					
cfu/mL initial	$4.6X10^5$	$1.55X10^5$	$2.05X10^5$	$2.03X10^5$	$2.5X10^5$
Microbial count as % of initial					
Day 7	0.00	0.00	0.00	0.00	0.00
Day 14	0.00	0.00	0.00	0.00	0.00
Day 21	0.00	0.00	0.00	0.00	0.00
Day 28	0.00	0.00	0.00	0.00	0.00

C.A. = *Candida albicans* A.N. = *Aspergillus niger*
S.A. = *Staphylococcus aureus* E.C. = *Escherichia coli*
P.A. = *Pseudomonas aeruginosa* c.f.u.= Colony forming units

The preservative effectiveness test is official in the USP, BP and also IP 1996. According to the USP specifications, the fungal count in the formulation should remain at or below the initial concentrations during the first 14 days of the test, the bacterial counts should be reduced to ≤ 0.1% of the initial

concentration by the 14th day. During the remaining period of test, the concentration of each test microorganism should remain at or below these designated levels.

In our study, the bacterial as well as fungal counts, in the aqueous solutions, came down to zero at the end of the first week itself and thus met the requirements of the USP. Ciprofloxacin present in the formulation too might have killed the bacteria. This cannot be avoided since the USP suggests that the test be carried out in un-opened containers of the test preparation.

Since ciprofloxacin possesses no antifungal properties, *Candida albicans* as well as *Aspergillus niger* should have been inhibited or killed by the preservative. Additionally, absence of any additive with nutritive properties in the formulation might have helped in inhibiting the growth of the microbes.

In the case of the ointment, a few fungal and yeast cells managed to survive, but their counts were well within the official limits.

2.5.3 *IN-VIVO* EVALUATION:

The development of a new product warrants preclinical evaluation. Animal models are an extremely useful tool in new product development. The model thus developed is such that it resembles the proposed human condition as closely as possible. The various studies that were carried out in animals included:

2.5.3.1 THE EYE-IRRITATION TEST:

The Draize test developed in 1944 is still recognized as the official test for assessing the eye-irritation potential of substances intended to be applied locally to the eye. The test substance is applied to the eye and the reaction of the ocular tissues are observed. An irritant would cause symptoms, such as reddening of the conjunctiva, increased tear flow and increased blinking rate, which are graded based on their severity and also compared with a standard non-irritating substance such as normal saline.

EXPERIMENTAL:

Our study was conducted as follows: Four New Zealand white rabbits, weighing between 2-2.5 kg, of either sex were used for testing of each formulation. The test formulation was applied to one eye of each rabbit and the contralateral eye served as a control in which a non-irritating solution such as sterile normal saline was instilled at the same time as that of the test formulations. After instillation, the eyes were observed for any immediate reaction and subsequently were observed on the morning of the next day. The following tissue response parameters were scored: light reflex, corneal damage (area of cornea affected), intensity of corneal damage, presence of cells in the anterior chamber, flare and hyperaemia of the iris, tear flow, conjunctival redness and conjunctival discharge. Depending on the severity of these responses, grades were allocated, the least severe reaction received the lowest grade. The dosing schedules

for the various formulations were as follows:

(i) Ciprofloxacin ophthalmic solution (the developed as well as the marketed formulation) : Day 1: 2 drops, every 30 minutes for the first 2 hours, then four doses every hour.(Total of 8 doses). Days 2-5: 2 drops every 2 hours (total of 6 doses).

(ii) Ciprofloxacin ophthalmic ointment: ~ 1cm was extruded out of the tube applied every 4 hours. (Total of three doses per day).

(iii) Ciprofloxacin ophthalmic gel: 2 drops from the vial every 4 hours (Total of 3 doses per day).

(iv) Ciprofloxacin with dexamethasone suspension and solution: Same as ciprofloxacin ophthalmic solution.

RESULTS & DISCUSSION:

All the formulations tested were found to be essentially non-irritating to the eye. The Draize test enlists eight different parameters to assess the eye irritation potential, as mentioned above. However, in our case there was so little irritation that only two parameters could be scored namely conjunctival redness and increased tear flow. The solution of CipHCl is acidic in nature and on instillation into the eyes it leads to slight reflex tearing in order to neutralize the instilled drop and this also causes transient conjunctival redness. This redness disappeared in about 5 min after instillation of the drops.

The gel on the other hand did not show any irritation potential because the instilled cold drop gelled in the eye and the drug was released over a period of time, this did not tax the buffering capacity of the tears, as whatever drug was released, was neutralized by the tears present in the eye.

The ointment was made with white soft paraffin base only as it is known to be non-irritating to the eyes. One other paraffin base,

which incorporated 3% w/w cholesterol was evaluated for its eye-irritation potential. In this base, cholesterol was incorporated since it would increase water uptake when applied in the eye and thus facilitate drug release. However, this base proved to be too irritating to the rabbit eyes and was therefore discarded.

If the formulation proves to be irritating to the eyes, it not only causes discomfort to the already distressed patient but also causes immense reflex tearing which would wash off all the instilled drug from the eye. Thus the prepared formulation should be evaluated for its eye irritation potential.

The Cip+Dexa suspension too proved to be non-irritating. Suspended drug particles if larger than 50µm in size can cause significant eye irritation upon instillation and may thus lead to excessive reflex tearing and discomfort. This formulation contained particles in the size range 3-7µm, and hence did not show any tendency to cause irritation.

The Cip+Dexa solution too did not exhibit any potential to cause eye irritation. Complexation of drugs with cyclodextrins have been reported to eliminate or decrease the eye irritation potential of the drug[251].

Corneal and iris injury are considered more relevant to the overall irritation potential and it was proposed by Bayard *et al*[252] to weigh the daily scores by using a multiplier of 15 for corneal damage score, a multiplier of 5 for iris scores and a multiplier of 2 for the conjunctival scores. The total score was simply the sum of scores of all the days on which the

preparations were instilled or applied. The total scores were evaluated using the following scale:

Severely irritant: 326-550 Strong irritant: 201-325

Moderately irritant: 66-200 Marginally irritant: 65 .

In our study, the total scores were never above 20, which is much below 65, the score of a marginally irritant substance. Hence all the prepared formulations were considered to be non-irritating.

2.5.3.2 EFFICACY STUDY OF CIPROFLOXACIN AND DEXAMETHASONE EYE-DROPS:

EXPERIMENTAL:

An animal model of bacterial keratitis induced corneal vascularization was developed and the ability of the developed formulation to prevent the vascularization was established. The procedure for infecting the rabbit corneas was as follows: New Zealand white rabbits weighing between 2-2.5 kg of either sex with normal ocular anatomy were selected for the study. The rabbits were held in restrainers and were anaesthetized with an intramuscular injection of Ketamine (5 mg/kg) and Xylazine (25 mg/kg). The eye was anaesthetized locally using 4% xylocaine solution. Following loss of corneal reflex (indicative of complete anaesthesia), 20µL of broth containing 1000 cfu of *S. aureus* ATCC 6538P, in its log phase of multiplication was injected intrastromally using a tuberculin syringe and 30 gauge needle. The intrastromal injection led to formation of a corneal bleb. Treatment was initiated 8 hours after infection was induced. The treatment format is shown in Table-40:

TABLE-40: TREATMENT PROTOCOL FOR EFFICACY STUDY OF CIP+DEXA FORMULATIONS

Animal No.	Left eye	Right eye
1	Saline	Cip+Dexa suspension
2	Saline	Cip+Dexa suspension
3	Cip solution	Cip+Dexa suspension
4	Cip solution	Cip+Dexa suspension
5	Cip solution	Cip+Dexa suspension
6	Cip solution	Cip+Dexa suspension
7	Cip solution	Cip+Dexa solution
8	Cip solution	Cip+Dexa solution
9	Cip solution	Cip+Dexa solution
10	Cip solution	Cip+Dexa solution
11	Saline	Cip+Dexa solution
12	Saline	Cip+Dexa solution

The treatment was given as follows:

Day 1: 2 drops of either saline, ciprofloxacin conventional solution, or ciprofloxacin solution/suspension, every 30 minutes for the first 2 hours, then 2 drops every hour (total of 8 doses in a day).

Days 2 - 12: 2 drops every 2 hours, a total of 6 doses per day.

The response parameter observed in all the eyes was the occurrence or prevention of corneal neovascularization at the site of injection.

RESULTS & DISCUSSION:

The efficacy of Cip + Dexa eye-drops was studied in an rabbit eye model of *S. aureus* induced keratitis, leading to neovascularization.

The corneas of the infected rabbit eyes that were treated with sterile normal saline, underwent extensive neovascularization and scarring (Fig. 11). Those treated with ciprofloxacin developed mild neovascularization, at the site of infection. However, none of the eyes treated with the combination of ciprofloxacin and dexamethasone developed this disorder, during the test period.

Acute infections of the cornea may lead to severe keratitis, which may result in neovascularization. This phenomenon occurs since the cornea is not a vascularized tissue and in cases of such infections, the body needs to rush the WBCs to the cornea, in order to combat the infection. Thus the limbal blood vessels penetrate the cornea to facilitate their transport. These WBCs, more precisely, the polymorphonuclear leukocytes, contain histolytic enzymes and when they breakdown in the corneal tissue, they cause local scarring, thus permanently impairing the vision. This is an immune reaction to an infection and the most potent agents that can prevent this are the steroids. By administering steroids along with antibiotics we not only eradicate the infecting bacteria, but also suppress the destructive inflammatory immune reaction. This point was proved by the prevention of corneal vascularization in infected eyes treated with ciprofloxacin as well as dexamethasone. The prevention of neovascularization by use of steroids has been evaluated in suitable animal models[253-255].

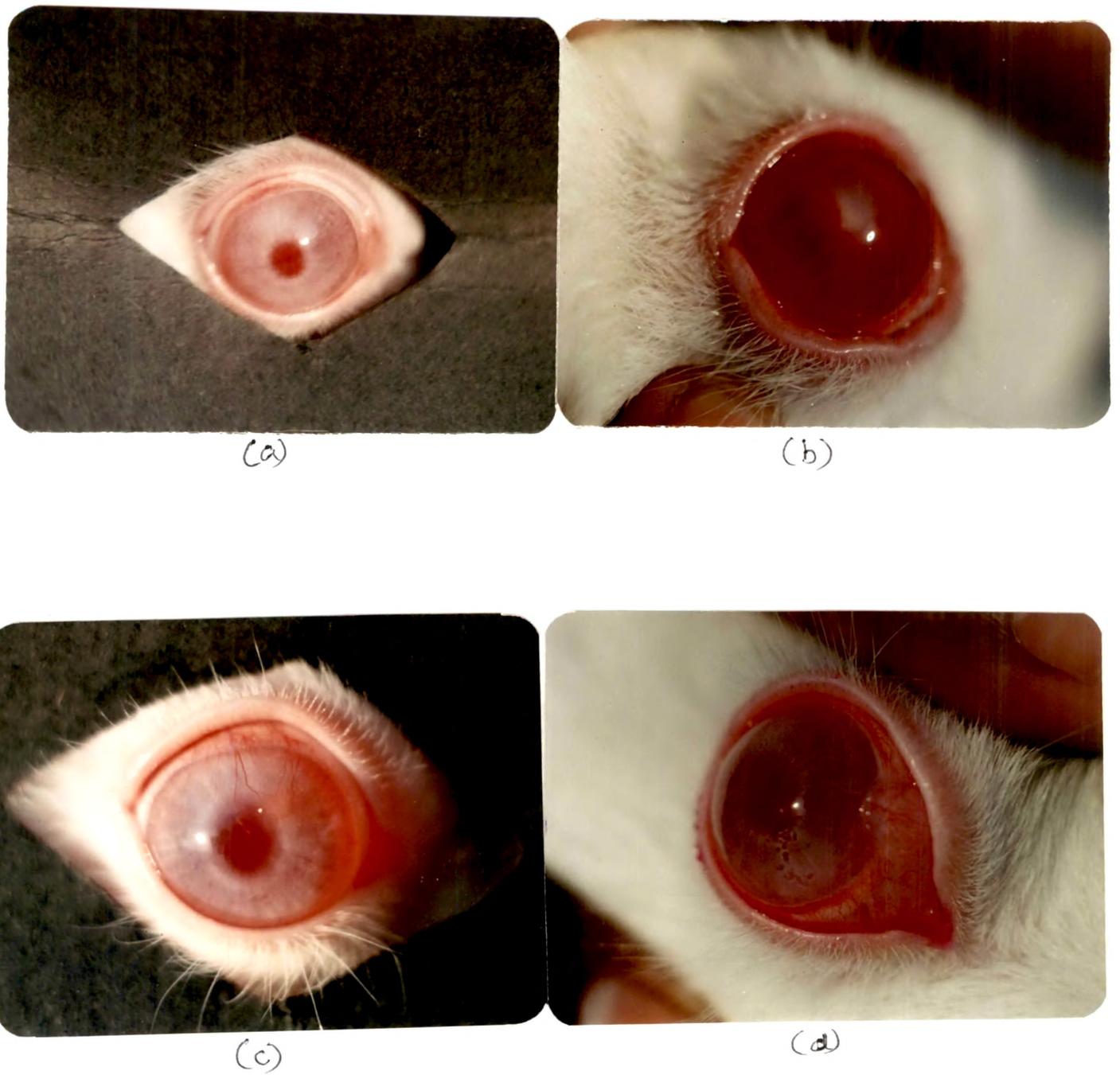

Fig. 11: Photographs of the rabbit eyes:
(a) normal eye (b) infected eye treated
with normal saline, (c) infected eye treated
with ciprofloxacin alone and (d) infected
eye treated with ciprofloxacin and dexamethasone

2.5.3.3. DETERMINATION OF THE AQUEOUS HUMOUR DRUG CONCENTRATION TIME PROFILE FOLLOWING APPLICATION OF A SINGLE DOSE OF FORMULATIONS CONTAINING CIPROFLOXACIN ALONE:

Pharmaceutical bioavailability/bioequivalence studies are carried out in human volunteers by administering a formulation of the drug under investigation, withdrawing blood samples and determining the plasma drug concentration time profile. The assumption is that the plasma is in dynamic equilibrium with all the tissues of the body. Such studies give a clear picture of the drug concentrations that may be achieved in the plasma and also tell us whether such profiles of the two formulations being compared, are superimposible, which will establish the bioequivalence. Similarly, it was thought that topical ophthalmic products too can be compared if the aqueous humour were to be assumed to be in dynamic equilibrium with the cornea, which is the target tissue in keratitis. This can be done by simply instilling the dose in the eyes of a set of rabbits and then withdrawing the aqueous humour over a period of time to estimate the drug levels in it.

EXPERIMENTAL:

The following three studies were conducted:

(1) Determination and comparison of the aqueous humour drug concentration-time profile after instillation of a single dose of Cip solution, and the marketed formulation Ciplox^{R}:

The study was carried out in 15 New Zealand albino rabbits, weighing between 2-2.5kg, of either sex and having normal ocular anatomy. These animals were divided into 5 groups of 3 animals each. One group of animals was sampled at only one time point

(sparse sampling)[256]. In order to dose the animals, they were placed in restrainers and the lower eyelid was pulled away to form the cul-de-sac. 50µL (equivalent to about 2 drops) of either formulation was carefully instilled in the cul-de-sac with the help of a micropipette and then the eyelids were closed for a period of 30 seconds. The dosing and sampling protocol was as follows:

TABLE-41: DOSING AND SAMPLING PROTOCOL FOR DETERMINING THE AQUEOUS HUMOUR DRUG CONCENTRATION - TIME PROFILE:

Animal No.	Group	Left eye	Right eye	Sampled at
1, 2, 3	I	Cip solution	Ciplox^R	15 min.
4, 5, 6	II	Cip solution	Ciplox^R	30 min.
7, 8, 9	III	Cip solution	Ciplox^R	60 min.
10, 11, 12	IV	Cip solution	Ciplox^R	120 min.
13, 14, 15	V	Cip solution	Ciplox^R	240 min.

The animals were anaesthetized with a mixture of ketamine (5mg/kg) and xylazine (25mg/kg), 10 minutes prior to withdrawal of sample. The eye was anaesthetized locally by instilling 2 drops of 4% xylocaine solution. The eye was then washed thoroughly with normal saline and blotted dry with a tissue paper. The anterior chamber was penetrated with the help of a 30 gauge needle and ~200µL of the aqueous humour was aspirated. The

aspirated sample was transferred to small glass conical tubes, sealed with parafilm and frozen to -20°C till the day of analysis. The samples were analyzed by the method described in Section 2.3.1.1.(c).

Before using the animals for further studies, they were allowed to rest for a period of 1 week.

(2) Determination and comparison of the aqueous humour drug concentration - time profile following topical application of multiple doses of Cip solution and single dose of Cip gel:

The number of animals and the criteria for selection was the same as described in the above experiment (1). For the purpose of dosing, 50µL of the conventional solution and 50µL of the cooled liquefied gel were instilled with the help of a micropipette. The sampling and dosing schedules were as shown in Table-42.
The sample collection, storage and assay procedures were the same as described in (1).

TABLE-42: DOSING AND SAMPLING PROTOCOL FOR DETERMINATION OF THE AQUEOUS HUMOUR DRUG CONCENTRATION - TIME PROFILE FOLLOWING APPLICATION OF MULTIPLE DOSES OF CIP. SOLUTION AND SINGLE DOSE OF CIP. GEL:

Animal No.	Group	Left eye (Cip. solution)		Right eye (Cip. gel)	
		Dosed at	Sampled at	Dosed at	Sampled at
1, 2, 3	I	0, 0.5 h	0.75 h	0 h	0.75 h
4, 5, 6	II	0, 0.5, 1.0, 1.5 h	1.75 h	0 h	1.75 h
7, 8, 9	III	0, 0.5, 1.0, 1.5, 2.5 h	2.75 h	0 h	2.75 h
10, 11, 12	IV	0, 0.5, 1.0, 1.5, 2.5, 3.5 h	3.75 h	0 h	3.75 h
13, 14, 15	V	0, 0.5, 1.0, 1.5, 2.5, 3.5, 4.5, 5.5 h	5.75 h	0 h	5.75 h

(3) Determination and comparison of aqueous humour drug concentration-time profile of ciprofloxacin following application of a single dose of Cip ointment and multiple doses of Cip solution:

The number of animals and the criteria for selection was the same as described in the above experiment (1). For the purpose of dosing, 50µL of the conventional solution was instilled with the help of a micropipette and 1cm ribbon of the ointment was applied with the help of a syringe, in which the ointment was filled. The sampling and dosing schedules were as shown in Table-43.

TABLE-43: DOSING AND SAMPLING PROTOCOL FOR DETERMINATION OF THE AQUEOUS HUMOUR DRUG CONCENTRATION - TIME PROFILE OBTAINED AFTER APPLICATION OF A SINGLE DOSE OF CIP. OINTMENT AND MULTIPLE DOSES OF CIP. SOLUTION.

Animal No.	Group	Left eye (Cip. solution)		Right eye (Cip. ointment)	
		Dosed at	Sampled at	Dosed at	Sampled at
1, 2, 3	I	0, 0.5 h	1.0 h	0 h	1.0 h
4, 5, 6	II	0, 0.5, 1.0, 1.5 h	2.0 h	0 h	2.0 h
7, 8, 9	III	0, 0.5, 1.0, 1.5, 2.5, 3.5, 4.5 h	5.0 h	0 h	5.0 h
10, 11, 12	IV	0, 0.5, 1.0, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5 h	7.0 h	0 h	7.0 h
13, 14, 15	V	0, 0.5, 1.0, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 8.5 h	9.0 h	0 h	9.0 h

The sample collection, storage and assay procedures were the same as described in experiment (1).

RESULTS & DISCUSSION:

Aqueous humour drug concentration time profile:

The results of the aqueous humour drug concentration - time profiles are shown graphically in Fig 12.

The drug concentrations in aqueous humour were found to be

comparable, after instillation of either Cip. solution or Ciplox^R, despite the high inter-subject variability. Since the study was based on sparse sampling, the Bailer's method[257,258] had to be applied for comparison of the AUCs. The AUCs were calculated using the trapezoidal rule. The calculated AUCs and variance AUCs for both formulations are shown below:

Parameter	Cip. Solution	Ciplox^R
AUC_{0-t}	292.44 ng.hr/mL	333.22ng.hr/mL
Variance AUC (s^2AUC)	706.84	7112.14

To test the null hypothesis H_o: $AUC_1 = AUC_2$ versus H_1: $AUC_1 \neq AUC_2$, the following statistic was used:

$$Z_{observed} = \frac{AUC_1 - AUC_2}{\{(s^2AUC_1) + (s^2AUC_2)\}^{\frac{1}{2}}}$$

H_o would be rejected if $|Z_{observed}| \geq Z_{critical}$, where $Z_{critical}$ is the critical value of the standard normal distribution. This test is an asymptotic two-tailed hypothesis test with significance level α. The results suggested that Ciplox^R and Cip. solution did not differ significantly ($0.3192 < P < 0.3228$) at the 5% level of significance.

The aqueous humour drug concentration - time profile after administering a single dose of the gel was compared to that obtained following multiple doses of the conventional solution. The data obtained is graphically represented in Fig. 13.

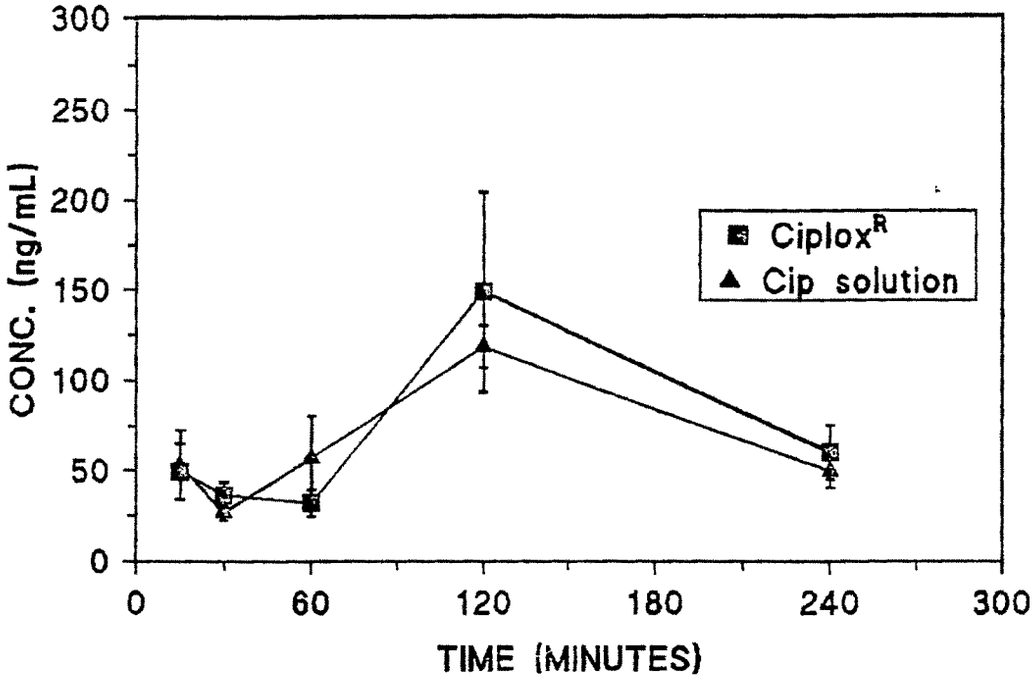

Fig. 12 Aqueous humour drug concentration–time profile following a single dose of Ciplox and the developed ophthalmic solution

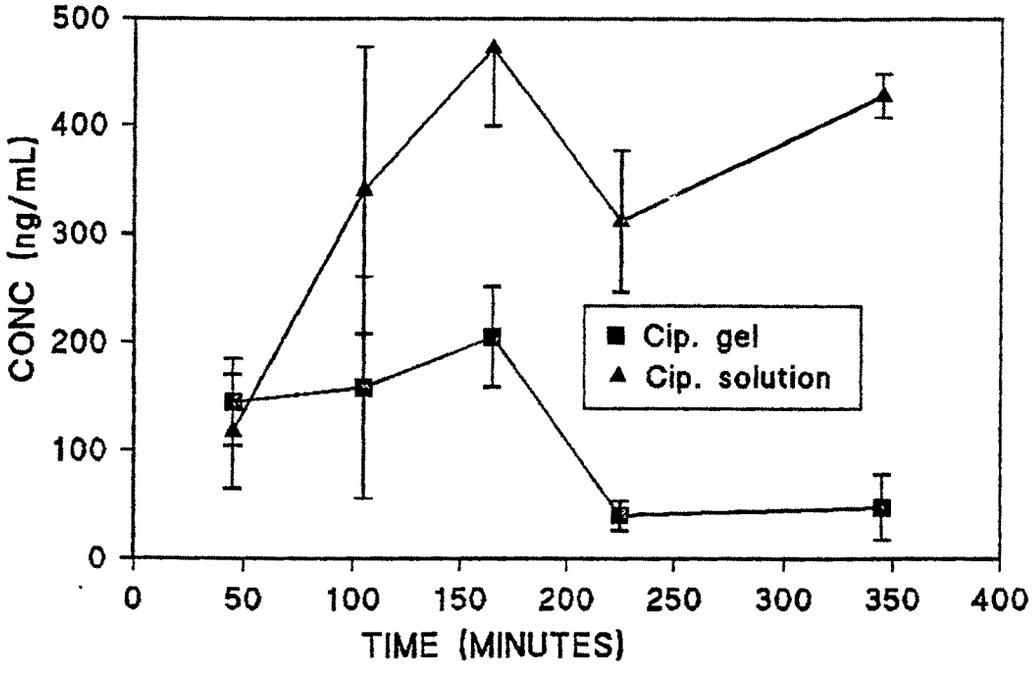

Fig. 13 Aqueous humour drug concentration–time profile obtained after instillation of multiple doses of solution and single dose of the gel

The drug concentrations obtained after instillation of a single dose of the gel were much higher than that obtained after single instillation of Cip. solution at 45 min, 105 min as well as at 165 min. The levels dropped sharply at 225 min and 345 min and at these time points were comparable to those of the solution.
These results indicate that higher concentrations are maintained for almost 3 hours, which would help in killing the infecting bacteria at a much faster rate, than that of the conventional solution. Thus providing the same dose of CipHCl in the form of a gel did increase its penetration into the cornea.
On dosing the conventional solution repeatedly, a steady increase in the aqueous humour drug concentration was observed, which justifies the frequency of instillation of the drops. However, with multiple dosing of the conventional solution, levels rise at a slow rate, but with a single dose of the gel, high concentrations were attained much faster.

The aqueous humour drug concentration - time profile obtained after instillation of multiple doses of the solution and application of a single dose of the ointment are shown graphically in Fig. 14. It was apparent that drug accumulated in the aqueous humour on multiple dosing of the conventional solution, which is essential for killing the bacteria.
The various pharmacokinetic parameters obtained for Cip. solution, Cip. gel and Cip. ointment are enlisted in Table-44.

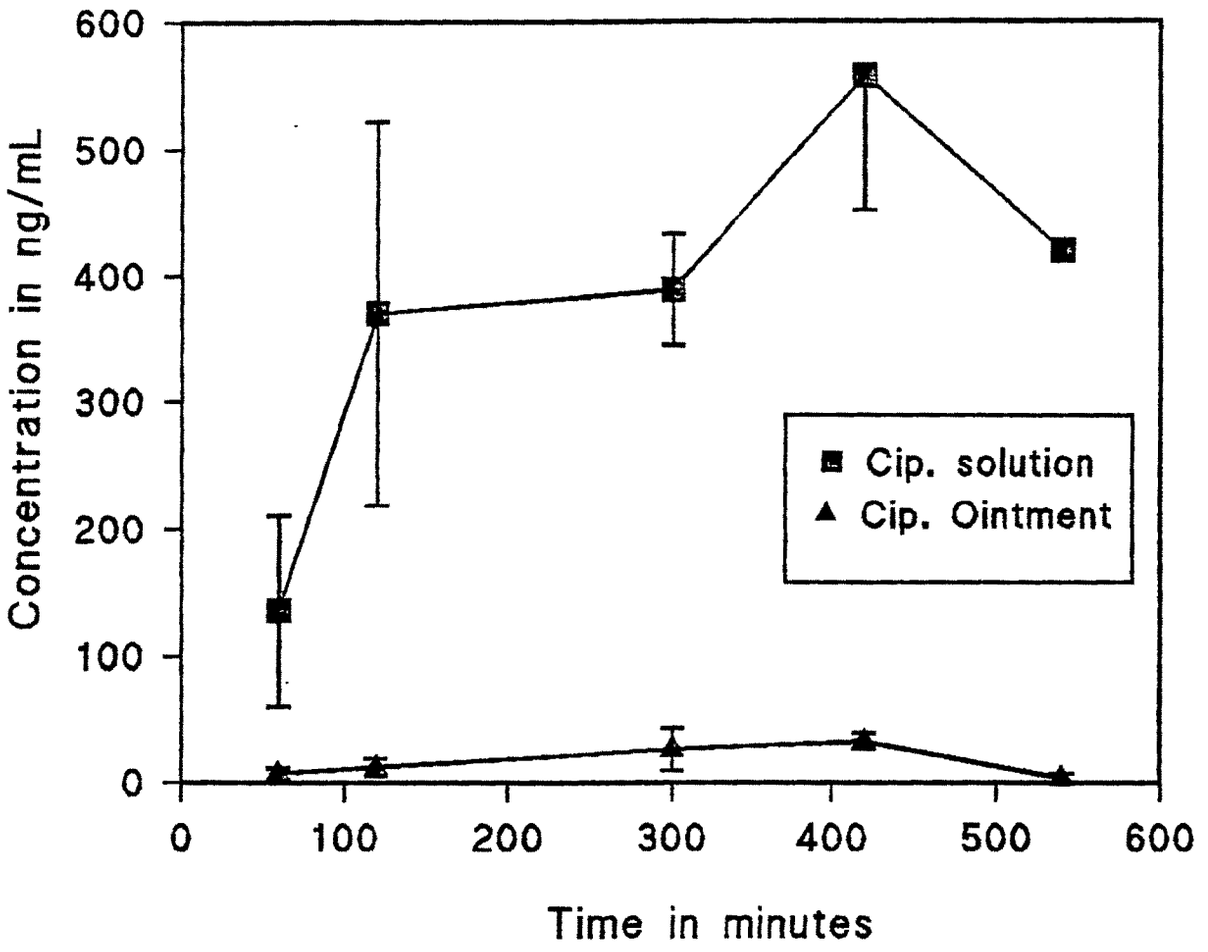

Fig. 14

Aqueous humour drug concentration – time profile obtained after application of single dose of ointment and multiple doses of solution

TABLE-44: RESULTS OF THE PHARMACOKINETIC VALUES OBTAINED FOR DIFFERENT DOSAGE FORMS:

Dosage form	C_{max}	T_{max}	AUC_{0-t}
Cip. solution	118.31ng/mL	2.00 h	292.44ng.hr/mL
Cip. gel	205.19ng/mL	2.75 h	720.14ng.hr/mL
Cip. ointment	32.83ng/mL	7.00 h	128.18ng.hr/mL

Extremely low levels were obtained after application of the hydrophobic ointment and the levels did not increase appreciably even after 9 hours of dosing (Table-43). This could have occurred because of (i) the inadequate wetting of the paraffin base by the tears and (ii) expulsion of a major part of the ointment due to blinking. The data suggests that the ointment should be used only as supportive therapy along with the conventional drops, eg. for application at bed-time.